

Denise M. Harmening

Clinical Hematology and Fundamentals of Hemostasis

Sixth Edition

With LeAnne M. Hutson



F.A. DAVIS

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Clinical Hematology and Fundamentals of Hemostasis

Sixth Edition

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INTRODUCTION TO CLINICAL HEMATOLOGY

CHAPTER 1

Morphology and Maturation of Human Blood Cells

Hematopoiesis

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This chapter is dedicated to the memory of a dear friend, Ann Bell, an excellent hematology professor and morphologist, and an extraordinary individual.

CHAPTER OUTLINE

Basic Morphology and Basic Concepts

Morphology of Cells on the Normal Blood Smear

Erythrocytes (Red Blood Cells)
Platelets (Thrombocytes)
Leukocytes (White Blood Cells)

Hematopoiesis

Description
Origin of Hematopoiesis

Erythropoiesis

Pronormoblast (Rubriblast,
Proerythroblast)
Basophilic Normoblast (Prorubricyte,
Basophilic Erythroblast)
Polychromatophilic Normoblast
(Rubricyte, Polychromatophilic
Erythroblast)
Orthochromatic Normoblast
(Metarubricyte, Orthochromatic
Erythroblast)

Reticulocyte (Diffusely Basophilic
Erythrocyte, Polychromatophilic
Erythrocyte)
Erythrocyte (Red Blood Cell, Discocyte)

Myelopoiesis (Granulocytopoiesis)

Morphological Changes
Stages of Differentiation and
Maturation

Monopoiesis

Monoblasts and Promonocytes
Monocytes and Macrophages

Lymphopoiesis

Lymphoblasts and Prolymphocytes
Lymphocytes
Plasmablasts and Proplasmacytes
Plasmacytes (Plasma Cells)

Megakaryocytopoiesis

Bone-Derived Cells
Osteoblasts

Osteoclasts

Cell Line Ontogeny (Evolution)

Multipotent Stem Cells—Colony-
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Recombinant Cytokines
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Cytokines
Clusters of Differentiation
Nomenclature
Clinical Applications of Cell Surface
Markers

Summary Chart

Case Study 1–1

Case Study 1–2

Review Questions

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LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 1-1 Describe the composition of human blood.
- 1-2 Identify the blood cells found on a normal peripheral smear.
- 1-3 Describe the two main differences between neutrophilic band and neutrophilic segmented cells.
- 1-4 Distinguish between eosinophils and basophils.

- 1-5 List four characteristics of lymphocytes.
- 1-6 Describe four morphological features that are helpful in identifying monocytes.
- 1-7 Compare and contrast a large lymphocyte with a monocyte.
- 1-8 List the proper cell maturation sequence of the erythroid series.

Continued

LEARNING OBJECTIVES—cont'd

- 1-9 List the proper cell maturation sequence for myelopoiesis (granulocytopoiesis).
- 1-10 Differentiate the morphological features of promyelocyte, neutrophilic myelocyte, neutrophilic metamyelocyte, and neutrophilic band.
- 1-11 Evaluate the morphology of a myeloblast.
- 1-12 Describe three features of a mature plasmacyte.
- 1-13 Describe hematopoiesis in the fetus.
- 1-14 Define the role of cytokines.

The primary step in assessing hematologic function and identifying the presence of disease is an examination of the cellular elements in the blood. More than 500 billion blood cells are produced every day in a process called hematopoiesis.¹ This chapter explains hematopoiesis and describes the stages of maturation of each blood cell, with an emphasis on the morphological characteristics and lineage-specific differentiation of each stage.

Basic Morphology and Basic Concepts

Hematology is the study of blood and its related disorders. Its birth can be traced to 1642, when Anton van Leeuwenhoek first noted cells in the blood. The significant milestones in the history of hematology are outlined in Box 1-1.

The average blood volume in an adult is 4 to 6 L; women have 4 to 5 L, and men 5 to 6 L.² This blood volume represents about 8% of the total body weight. Blood has a pH between 7.35 and 7.45. Blood is composed of 55% plasma (the fluid portion) and 45% formed elements or cells. Of the 45% cellular elements, approximately 44% of the cells are red blood cells (RBCs), whereas only 1% are white blood cells (WBCs) and platelets (PLTs) (Fig. 1-1). Plasma is composed of about 91.5% water, 7% proteins, and 1.5% other solutes. The solutes consist of three different kinds of proteins: albumins (55%), globulins (38%), and fibrinogen (7%); other solutes are electrolytes, hormones, nonprotein nitrogen compounds, nutrients, and respiratory gases.² The reference values for the cellular elements are as follows: RBCs (4.2 to $5.4 \times 10^{12}/L$ for females and 4.7 to $6.1 \times 10^{12}/L$ for males), WBCs (4.8 to $10.8 \times 10^9/L$), and PLTs (150 to $400 \times 10^9/L$) in adults.² These reference values vary with age, gender, geographic location, and health or disease.

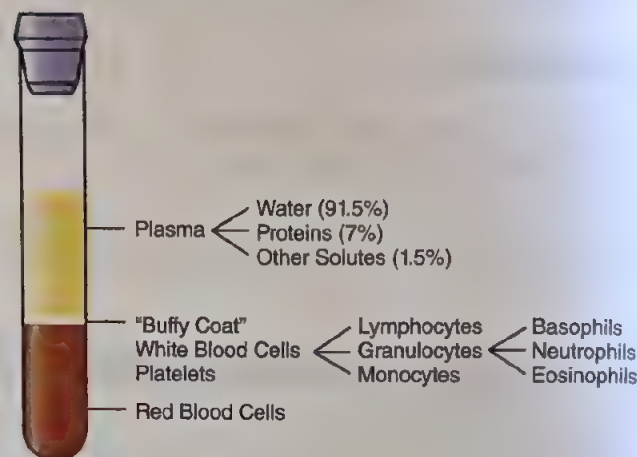


FIGURE 1-1 Centrifuged whole blood depicting plasma, buffy coat, and RBC layers.

Morphology of Cells on the Normal Blood Smear

Examination of the blood frequently elicits important information that aids in the diagnosis of hematologic disease and may suggest further testing. A well-made and well-stained blood smear is vital, because the analysis of cell morphology may be greatly hindered by poorly made and poorly stained smears. (See Chaps. 4 and 31 for smear preparation.)

Careful examination of cell morphology on a blood smear and determination of the percentage of each type of blood cell present is an important skill to master. Blood cells normally present on a blood smear are RBCs (erythrocytes), white blood cells (leukocytes), and platelets (thrombocytes) (Fig. 1-2). Morphological descriptions of each of these cellular elements in normal blood are presented in this chapter.

Erythrocytes (Red Blood Cells)

Erythrocyte morphology is evaluated in an area of the stained smear where red cells are evenly distributed and do not overlap (Fig. 1-3). Red cells consist of a plasma membrane surrounding a solution of proteins (mainly hemoglobin) and electrolytes. A normal mature erythrocyte is a biconcave disc that is 7 to 8 μm in mean diameter and 1.5 to 2.5 μm thick. The RBC has a mean volume of 90 femtoliters (fL). After the smear is stained with Wright's stain, an erythrocyte appears as a circular cell with distinct and smooth margins and a dull pinkish hue. In the central portion of the erythrocyte where the cell is thinnest, the intensity of the stain is less than at the marginal area, creating an area of central pallor. Red cells should

BOX 1-1 History of Hematology

Date	Key Milestones
1642	van Leeuwenhoek first notes cells in blood.
1842	Donne discovers platelets.
1846	Gulliver differentiates lymphocytes from granulocytes by size.
1874	Malassez counts white blood cells (WBCs) via hemocytometry.
1875	Hayem defines methods for counting platelets.
1879	Ehrlich uses aniline dyes to stain WBCs.

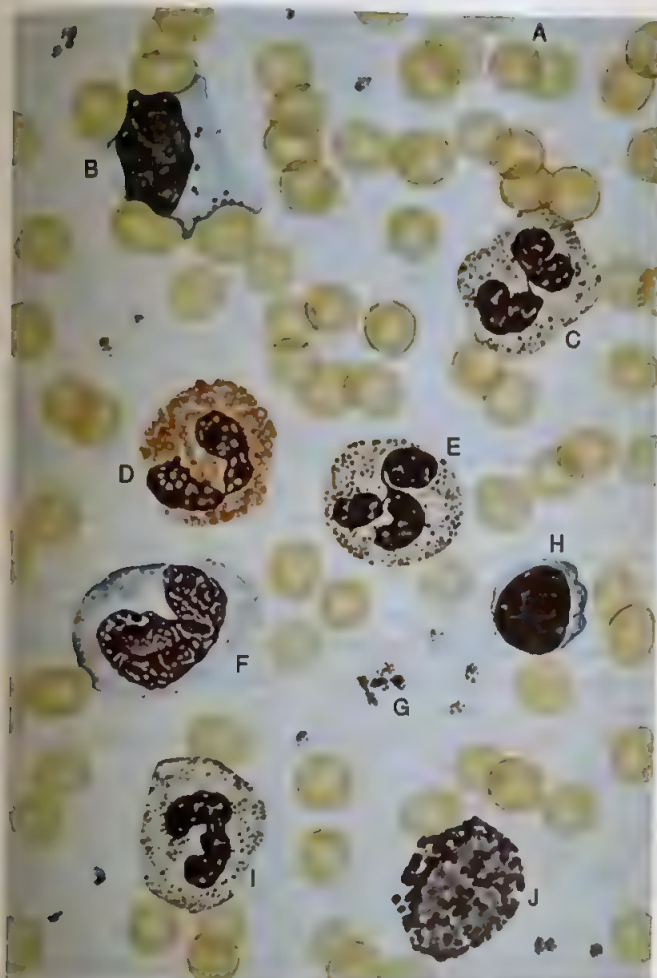


FIGURE 1-2 Cell types found in smears of peripheral blood from normal individuals. A. Red blood cells (RBCs). B. Large lymphocyte. C. Segmented neutrophil. D. Eosinophil. E. Segmented neutrophil. F. Monocyte. G. Platelets. H. Small lymphocyte. I. Neutrophilic band. J. Basophil.

be fairly uniform in size and relatively round in shape, with a small area of central pallor and no nucleus or inclusions.

Platelets (Thrombocytes)

In the same area where erythrocyte morphology is being studied, the number of platelets per oil immersion field and the morphology should be evaluated. Platelets are approximately 1 to 4 μm in diameter and vary in shape.² An average of 7 to 15 platelets per oil immersion field is normally observed.³ An estimate of the number of platelets in 10 oil immersion fields should be made.³ Platelets may be observed in small groups (see Fig. 1-2G). A count of individual platelets in a group should be made.²

In Wright's stain, a platelet contains reddish-purple granules in a small amount of bluish cytoplasm, but there is no nucleus. Platelets contain particular molecules needed for hemostasis and are able to adhere, aggregate, and supply a surface for coagulation reactions.²

Leukocytes (White Blood Cells)

The morphology and the distribution of leukocytes are routinely evaluated. WBCs normally observed on a blood smear include neutrophils, eosinophils, basophils, lymphocytes,

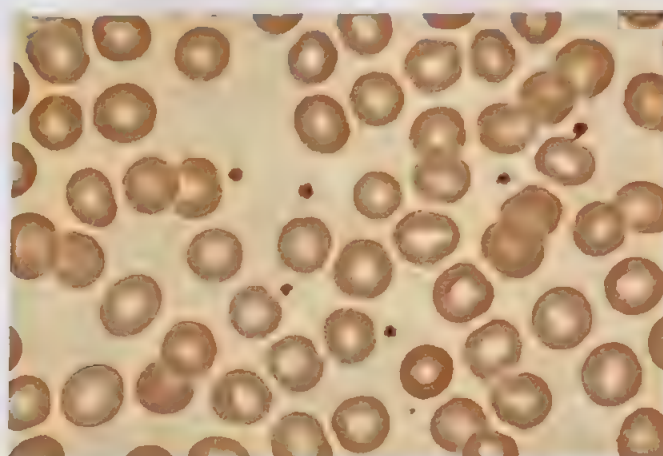


FIGURE 1-3 Normal peripheral blood smear; normal erythrocytes and platelets.

and monocytes (see Fig. 1-2). Immature cells of any type are abnormal. Cells should be examined for abnormalities in nucleus or cytoplasm.

Segmented Neutrophils (Filamented Neutrophils, Polymorphonuclear Neutrophils)

In normal peripheral blood of older children and adults, 50% to 70% of mature **granulocytes** called **segmented neutrophils** are found, making it the most common type of WBC.⁴ Table 1-1 summarizes the percentage and absolute values of cells found in the peripheral blood of normal adults. The nucleus of the segmented neutrophil is separated into two to five (usually three) lobes, with a narrow segment or **filament** connecting the lobes³ (Fig. 1-4). Approximately 6% of the neutrophils have one lobe (band neutrophil), 35% have two lobes, 41% have three lobes, 17% have four lobes, and 2% have five lobes.³ Segmentation of the nucleus enables these motile cells to pass through an opening in capillary endothelial lining cells and “home in” on selected prey (such as microorganisms causing infection).⁴ Neutrophils' nuclear chromatin is heavily clumped, coarse, or **pyknotic** and stains purplish-red (see Figs. 1-2C and 1-2E).³ The cytoplasm is light pink when stained properly, and the secondary granules (which are fine, numerous, and evenly distributed) stain either pink or a neutral color. Neutrophil secondary granules are lysosomes that contain alkaline phosphatase.²

Neutrophils (also called granulocytes) as well as monocytes play a key role in inflammation and phagocytosis. They

TABLE 1-1 Peripheral Blood Cells: Normal Adult Values

Type of Cell	Percentage	Absolute Values (per mm^3)
N. band	2-6	100-650
N. segmented	50-70	2400-7500
Eosinophil	0-4	0-450
Basophil	0-2	0-200
Lymphocyte	20-44	1200-3400
Monocyte	2-9	100-900



FIGURE 1-4 Two segmented neutrophils.

migrate from the blood vessel into the tissues, where they serve as the first line of defense against bacterial infections.

Band Neutrophils (Nonsegmented Neutrophils, Nonfilamented Neutrophils)

Peripheral blood of healthy individuals contains 2% to 6% of the **band neutrophils** (see Table 1-1). Band neutrophils have a nucleus with a horseshoe or sausage shape in which the opposite edges of the nucleus become almost parallel for an appreciable distance.³ These cells do *not* have a nucleus separated into lobes connected by a filament (Fig. 1-5).

The nuclear chromatin is clumped, and there is usually a dark pyknotic mass at each pole where the lobe is destined to be.³ The secondary neutrophil granules are small, evenly distributed, stain various shades of pink (on Wright's stain), and contain alkaline phosphatase. There may be an occasional dark primary granule (see Fig. 1-2f).³

Difficulty may arise in differentiating between band and segmented neutrophils and in deciding whether the link connecting the lobes is narrow enough to be called a filament or wide enough to be identified as a band. A filamented or segmented cell has a threadlike connection between two lobes, and there is no visible chromatin between the two sides of the filament. Lobes of nuclei often touch each other or overlap,

and it may be impossible to see the connecting filaments. In a band neutrophil, there are two distinct margins, with nuclear chromatin material visible between the margins. If the margin of a lobe can be traced as a definite and continuing line from one side of the nucleus across the isthmus to the other side, then it may be assumed that a filament is present even though it is not visible. In attempting to differentiate between a segmented and band neutrophil, identification should not be made on a single morphological characteristic but on combined features. In case of doubt regarding a borderline cell, the questionable cell should be placed into the *mature category*.³

Eosinophils

Eosinophils are usually easily recognizable because of the large, round, secondary, refractile granules that have an affinity for the acid eosin stain (see Figs. 1-2D and 1-6). With Wright's stain, normal eosinophilic granules become orange to reddish-orange. The granules are spherical, uniform in size, and evenly distributed. Because of the size and roundness of the granules, eosinophils may be recognized in unstained moist preparations of blood on light microscopy and via phase microscopy. The crystalloid core of the granule is composed mainly of major basic protein (MBP), which binds to acid aniline dyes and may help to explain the staining qualities of the granule.²

Normal adult peripheral blood contains 0% to 4% eosinophils. Normal blood eosinophils are about the size of or slightly larger than neutrophils and have a band or two-lobed nucleus with condensed chromatin; rarely does an eosinophil have three lobes. There is a diurnal variation in the percentage of circulating eosinophils, which increases at night and decreases in the morning.

Basophils

Although **basophils** constitute only 0% to 2% of normal blood cells, the large, abundant, violet-blue (or purple-black) granules aid in the immediate recognition of this cell. These granules are visible above the nucleus as well as lateral to it, and they obscure most of the nucleus. The granules vary in size from 0.2 to 1.0 μm .³ They are coarse and unevenly distributed; vary in number, shape, and color; and are less numerous than eosinophil granules (see Figs. 1-2J and 1-7).³ These granules



FIGURE 1-5 Neutrophilic band.



FIGURE 1-6 Eosinophil (segmented).

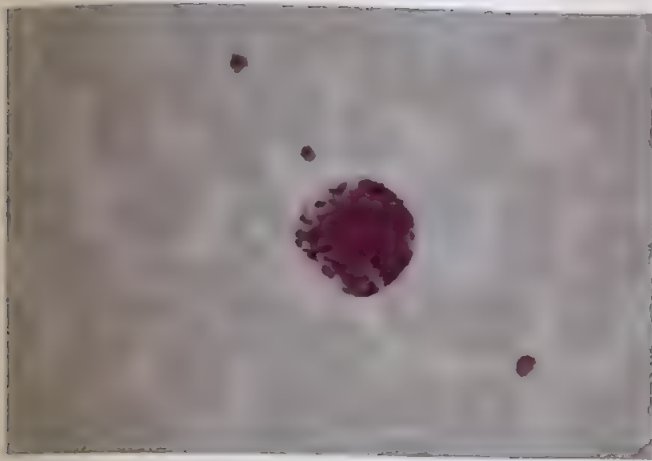


FIGURE 1-7 Basophil.

have an affinity for blue or basic thiazine dyes. Basophil granules are also water soluble. In cells that are poorly fixed during staining, the center of the granule may disappear or the entire granule may be washed away, leaving a small colorless cytoplasmic area.

Basophils show a diurnal variation similar to that of eosinophils, increasing at night and decreasing in the morning.³

Lymphocytes

Lymphocytes are the second most numerous cells in the blood, comprising from 20% to 44% of the adult blood cells. Most lymphocytes are small, varying from 7 to 10 μm . There are also intermediate sizes and some large lymphocytes (Fig. 1-8).³ Size is not a reliable basis for determining the age or metabolic activity of lymphocytes because their size varies with the thickness of the smear. Lymphocytes tend to become spherical and small in thick areas of the smear; in the thinnest end of the smear, lymphocytes may spread out and appear large.² Small lymphocytes are usually round with smooth margins (see Figs. 1-2H and 1-8A). Rarely, a lymphocyte may have a spindle form with an oval nucleus and cytoplasmic filaments extending outward at each end (see Fig. 1-8F). The margin of large lymphocytes frequently is indented by neighboring erythrocytes, causing them to have a serrated (holly leaf) shape³ (see Figs. 1-8J through L).

With Wright's stain, the color of the cytoplasm is blue, varying in intensity from light to dark in different cells and appears clear, not cloudy. The color is evenly distributed in some cells and uneven in other cells. Most lymphocytes do not have granules. In some large cells, there may be a few well-defined granules that vary in size, are unevenly distributed, and can be easily counted. These granules are a purplish-red and have been called *azurophilic*; however, the term is misleading because the granules are predominantly red rather than blue³ (see Figs. 1-8J, K and 1-9).

The diameter of the nucleus of a small lymphocyte with scant cytoplasm in peripheral blood is slightly larger than, or the same size as, a normal erythrocyte in the same microscopic field. The lymphocyte's nucleus, in relation to its cytoplasm, is large (N:C ratio is 4:1 to 2:1), and the nuclei are

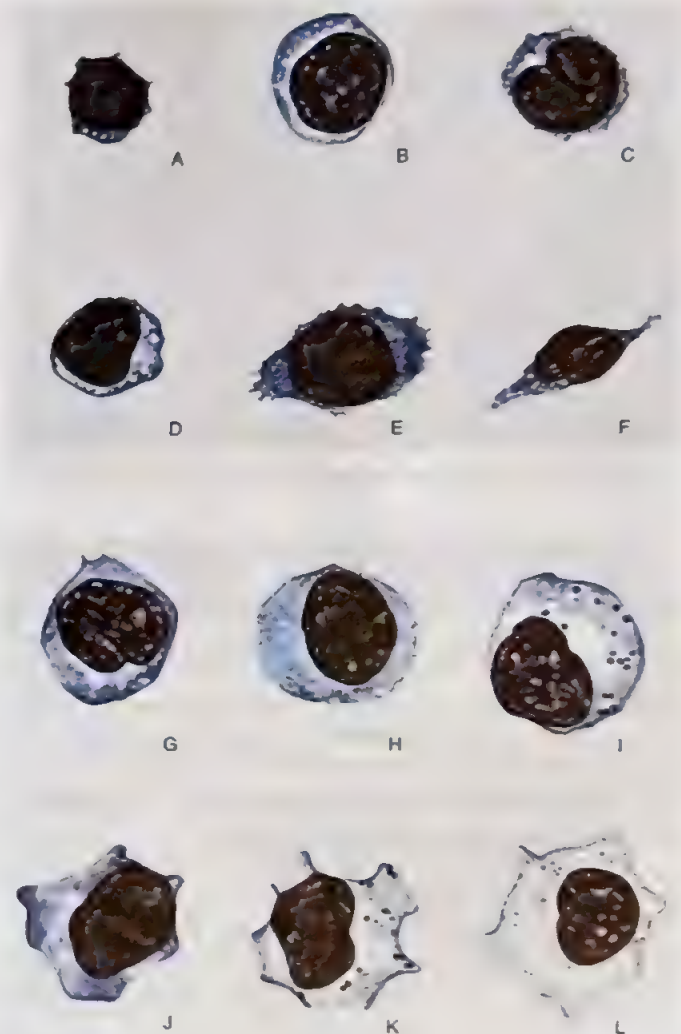


FIGURE 1-8 Lymphocytes. A. Small mature lymphocyte. B. Lymphocyte of intermediate size. C. Lymphocyte with indented nucleus. D. Lymphocyte of intermediate size. E. Lymphocyte with pointed cytoplasmic projections (frayed cytoplasm); typical nucleus. F. Spindle-shaped and pointed cytoplasmic projections. G. Large lymphocyte with indented nucleus and pointed cytoplasmic projections. H. Large lymphocyte. I. Large lymphocyte with purplish-red (azurophilic) granules. J. Large lymphocyte with irregular cytoplasmic contours. K. Large lymphocyte with purplish-red (azurophilic) granules and with indentations caused by pressure of erythrocytes. L. Large lymphocyte with purplish-red (azurophilic) granules. (From Diggs LW, et al. *The Morphology of Human Blood Cells*, ed 5, 1985, pp 1-18, 25-27. Abbott Laboratories, Abbott Park, IL, with permission.)

round or slightly indented. Chromatin structure is lumpy or clumped and stains dark purple with lighter bluish-purple areas between chromatin aggregates.³

Nucleoli are present in some lymphocytes but are not visible on light microscopy because they are obscured by the darkly stained chromatin masses.

Monocytes

In the thin areas of the peripheral blood smear, a monocyte measures about 12 to 18 μm and is larger than the mature neutrophil.⁴ Monocytes have abundant cytoplasm in relation to the nucleus (N:C ratio is 1:1 or 2:1).³ With Wright's stain, the cytoplasm turns a dull gray blue, in contrast to the pink

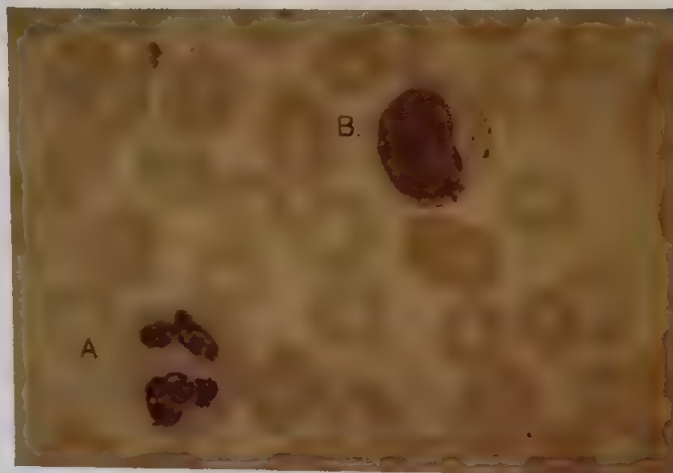


FIGURE 1-9 A. Segmented neutrophil. B. Lymphocyte with azurophilic granules.

cytoplasm of the neutrophils. Numerous fine, small, reddish- or purplish-stained, evenly distributed granules in the cytoplasm give the cell a ground-glass, cloudy appearance (see Figs. 1-2F and 1-10).³ There may be varying numbers of prominent granules in addition to the small granules. Some monocytes may appear nongranular, suggesting rapid turnover. Digestive vacuoles may be observed in the cytoplasm. In disease states, phagocytized erythrocytes, nuclei, cell fragments, bacteria, fungi, and pigment may be present.⁴

The nuclei of monocytes frequently may be kidney-shaped, deeply folded or indented, or occasionally lobular. One of the distinctive features of the monocyte is the appearance of **convolutions** (like those in the brain) in the nucleus (see Figs. 1-10 and 1-11).³ Another characteristic is the lacy, often

delicate chromatin network of intermingled fine strands with small chromatin clumps.

The shape of the monocyte is variable. Many cells are round; other cells reveal blunt **pseudopods** that are manifestations of their slow mobility. Pseudopods vary in size and number; the outer portion of the outstretched cytoplasm may have a hyaline appearance without granules, in contrast to the inner granular cytoplasm.

It is helpful to memorize four helpful characteristic features of the monocytes: nuclear convolutions; lacy, often delicate chromatin; dull gray-blue cytoplasm; and blunt pseudopods (see Fig. 1-10H). Kinetic studies have revealed that the half-life of monocytes in the circulation ranges from 8 hours to 3 days before these cells enter tissues and are transformed into macrophages.⁴ Monocytes account for 2% to 9% of normal blood leukocytes.⁴

Large Lymphocytes Versus Monocytes

A monocyte (see Fig. 1-10D) is often mistaken for a large lymphocyte (see Figs. 1-8G through L) because the monocytic cytoplasm may be blue, the granules may be indistinct, the nucleus is round, and the blunt pseudopods and digestive vacuoles are missing. To distinguish monocytes from large lymphocytes, it is useful to observe the nuclear chromatin structure, character of the cytoplasm, and shape of the cells. The nucleus of a lymphocyte tends to be clumped (Fig. 1-12), rather than linear or lacy as it is in a monocyte. There is a greater tendency for the nuclear chromatin to be condensed at the periphery of the nucleus in the lymphocyte. The brain-like convolutions present in a monocyte (Fig. 1-13) are *not* observed in a lymphocyte (Fig. 1-14).

Large lymphocytes and monocytes may have distinct bluish-red granules. In a monocyte, the large bluish-red

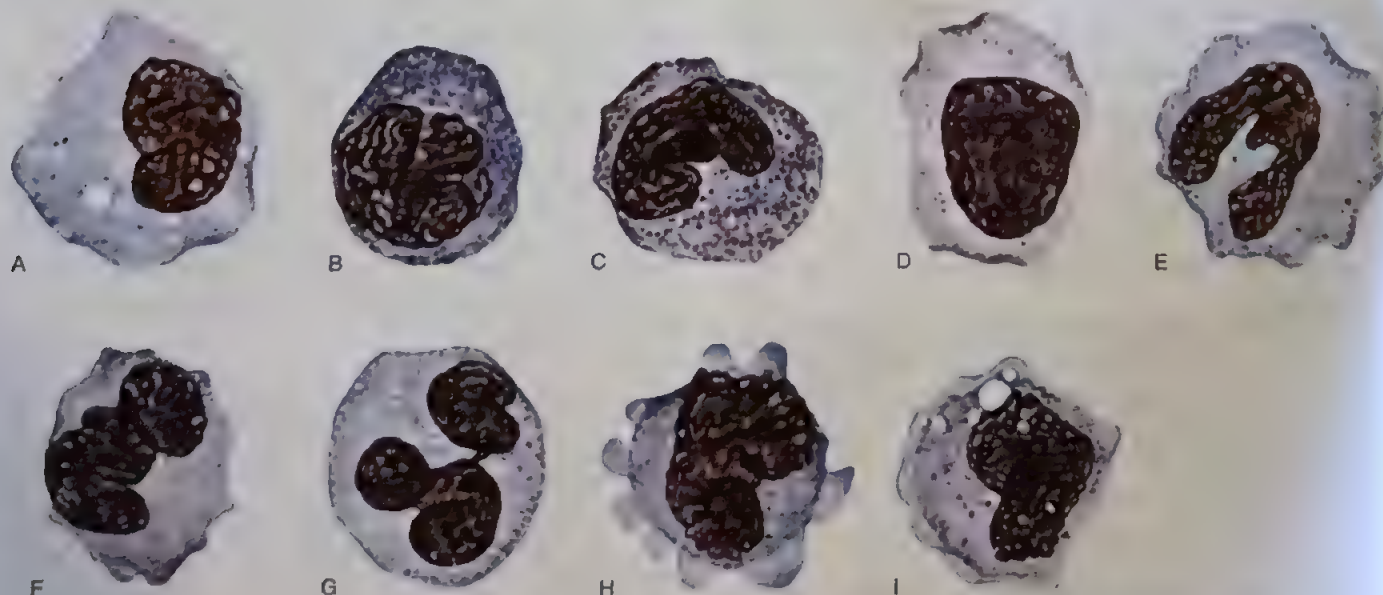


FIGURE 1-10 Monocytes. A. Monocyte with "ground-glass" appearance, evenly distributed fine granules, occasional azurophilic granules, and vacuoles in cytoplasm. B. Monocyte with opaque cytoplasm and granules and with lobulation of nucleus and linear chromatin. C. Monocyte with prominent granules and deeply indented nucleus. D. Monocyte without nuclear indentations. E. Monocyte with gray-blue color, band type of nucleus linear chromatin blunt pseudopods, and granules. F. Monocyte with gray-blue color, irregular shape, and multilobulated nucleus. G. Monocyte with segmented nucleus. H. Monocyte with multiple blunt nongranular pseudopods, nuclear indentations, and folds. I. Monocyte with vacuoles and with nongranular ectoplasm and granular endoplasm. (From Diggs, LW, et al. *The Morphology of Human Blood Cells*, ed 5, 1985, pp 1-18, 25-27. Abbott Laboratories, Abbott Park, IL, with permission.)

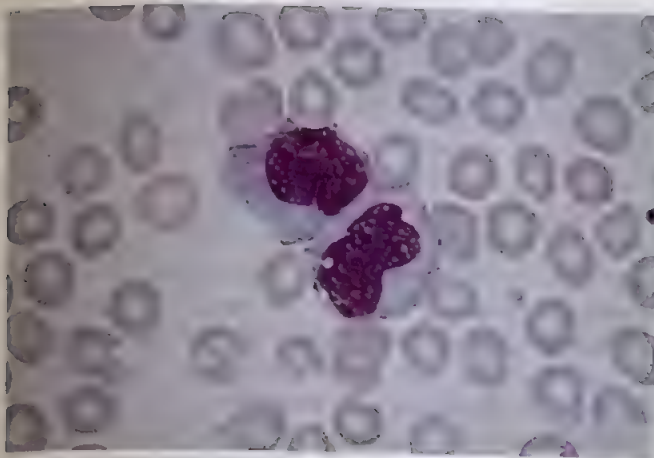


FIGURE 1-11 Monocytes.

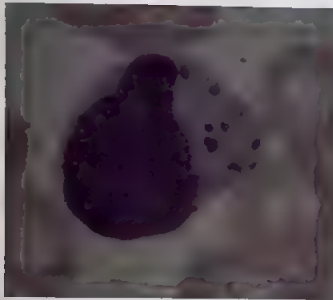


FIGURE 1-12 Lymphocytes with azurophilic granules.

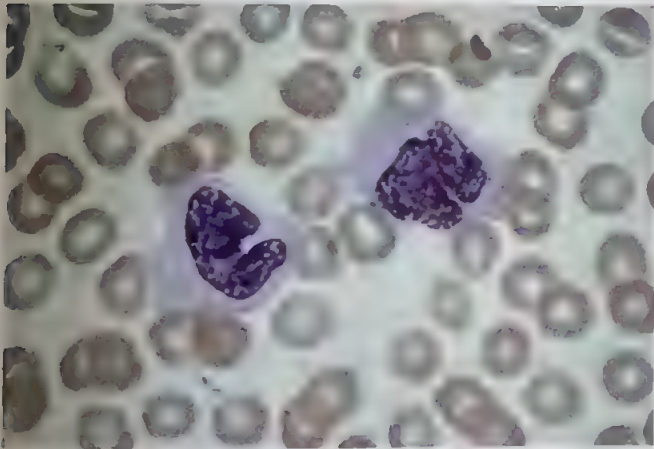


FIGURE 1-13 Two monocytes.

granules are interspersed with numerous fine granules in the cytoplasm and cannot be enumerated (see Figs. 1-10B and C). In a lymphocyte, these large granules are prominent (sometimes at the periphery of the cytoplasm) and can be counted easily because there are no other granules (see Figs. 1-8I and K). Because of the finely granular cytoplasm, the monocyte has a ground-glass appearance; the cytoplasm of the lymphocyte has a relatively clear, nongranular background. Large lymphocytes are often deeply indented by neighboring RBCs (see Fig. 1-8K). Monocytes tend to project blunt pseudopods between cells or to compress cells, rather than being indented by them. Table 1-2 presents a morphological comparison of large lymphocytes with monocytes.

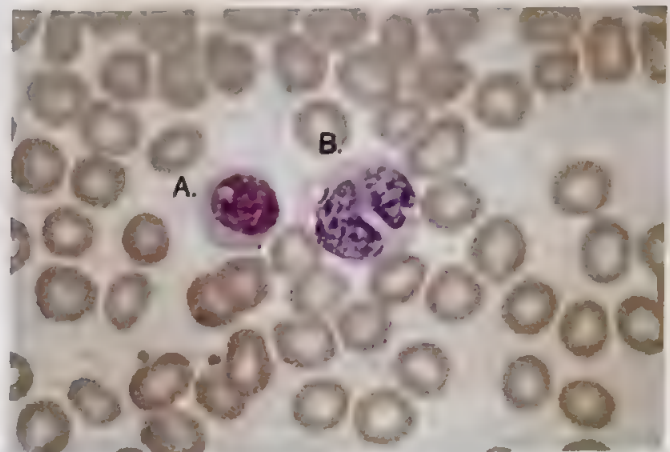


FIGURE 1-14 A. Lymphocyte. B. Monocyte.

TABLE 1-2 Morphological Comparison of Large Lymphocytes and Monocytes

Feature	Large Lymphocyte	Monocyte
Size, μm	12–15	12–18
Nucleus	Clumped, condensed at periphery	Lacy, brainlike convolutions
Cytoplasmic granules	Bluish-red, prominent granules, easily enumerated if present	Bluish-red, interdispersed with other granules, not easily enumerated
Cytoplasm	Clear, nongranular background	“Ground-glass” appearance (cloudy)
Cell interactions	Indented by erythrocytes	Projection of blunt pseudopodia

CRITICAL THINKING QUESTION

1-1 Which white blood cell would you expect to see increased in a patient with a bacterial infection? Would any variations exist if the infection was advanced?

See answers to all Critical Thinking Questions at the back of this book.

Hematopoiesis

Hematopoiesis is the name given to the dynamic processes of blood cell production and development of the various cells of the blood. Strong evidence exists that all blood cells are derived from hematopoietic stem cells.⁴

Description

Hematopoiesis is characterized by a constant turnover of cells. The normal hematopoietic system continuously maintains a cell population of erythrocytes, leukocytes, and platelets through a complex network of tissues, organs, stem cells, and regulatory factors.¹ This network is responsible for the maturation and division of **hematopoietic stem cells (HSC)** into the lineage-committed stages that transport oxygen and

excrete carbon dioxide (erythrocytes), fight infection (granulocytes), support cellular and humoral immunity (lymphocytes), and maintain hemostasis, a process in which blood clots and bleeding is halted (platelets).

ADVANCED CONTENT

- Hematopoiesis is usually depicted as a hierarchy, with HSCs giving rise to precursors that are committed to one or more pathways supported with a variety of cytokines. Cytokines involved in hematopoiesis include interleukins (ILs), colony-stimulating factors (CSFs), interferons, erythropoietin (EPO), and thrombopoietin (TPO).⁵
- In hematopoiesis, differentiation is well characterized and involves intermediate progenitors with decreasing self-renewal ability and increasing lineage commitment. Lineages are defined functionally and morphologically. Lineage commitment is controlled by a complex network of transcription factors that define specific gene expression patterns for every cell type.^{6,7,8} Transcription factors are involved in the regulation of cytokines.^{9,10} Transcription factors (TFs) recognize specific DNA sequences to control chromatin and transcription, forming a complex system that guides expression of the genes.¹¹

The majority of hematopoiesis occurs in the bone marrow and is derived from a limited number of hematopoietic stem cells (HSCs) that are multipotent and capable of extensive

self-renewal.⁵ HSCs reside in specialized microenvironments in the bone marrow called **niches**.^{6,12} The stem cell niche is thought to provide signals that support key HSC properties, including self-renewal capacity and long-term multilineage repopulation ability.^{13,14} It represents a highly regulated dynamic complex structure that maintains core properties of HSCs and **hematopoietic progenitor cells (HPCs)** in the steady state.¹⁵ The stem cell niches modulate the proliferation and differentiation of HSCs and HPCs in response to physiological demands.¹⁵ These properties of the niche are shared by the intuitive immune system in which immune cells, including macrophages and neutrophils, are now recognized as important regulators of the hematopoietic niche.¹⁵ The hematopoietic stem cell has the capacity for continuous self-replication and proliferation, together with the ability to differentiate into committed HPCs of lymphoid and myeloid lineages.¹⁶ Under the influence of growth factors (cytokines) such as colony-stimulating factors, cytokine stem cell factor (SCF), and interleukins, to name a few, progenitor cells divide and differentiate to form the mature cellular elements of the peripheral blood^{16,17} (see Fig. 1-15).

ADVANCED CONTENT

The extrinsic regulation of HSCs involve a variety of cell types including endothelial cells, mesenchymal stromal cells, neural cells, megakaryocytes, macrophages, and osteoblasts.¹⁸ The major regulatory cells are endothelial cells and mesenchymal stromal cells, which are required to maintain the HSC pool. The other regulatory cells, which include

FIGURE 1-15 Hematopoiesis Simplified. A pluripotent haemopoietic stem cell (HSC) can self-renew or differentiate into a multipotent progenitor stem cell (MPP). MPP differentiation produces common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). The CMP differentiates into the granulocyte monocyte progenitor (GMP) and the megakaryocyte-erythroid progenitor (MEP). The GMP differentiates to the granulocyte progenitor (GP) and monocyte progenitor (Mop). The MEP differentiates into the erythroid progenitors (Ep) and megakaryocyte progenitors (Mp). The CLP differentiates into B lymphocyte progenitor (Bp), T lymphocyte progenitor (Tp) and NK lymphocyte progenitor (NKp).

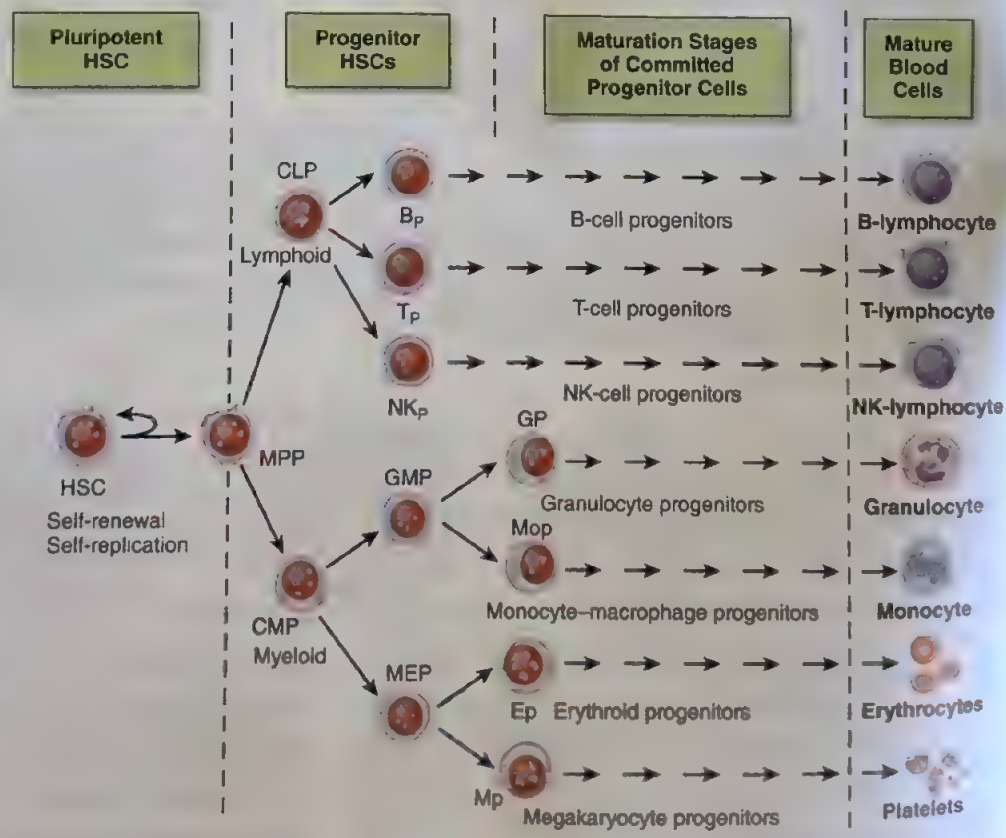


TABLE 1-3 Selected Cytokines Produced by Major and Other Regulatory Cells¹⁸

Major Regulatory Cells	Cytokines	
	Previous Name	Current Name
Endothelial	Stem cell factor (SCF)	CXCL 12
	Stromal cell-derived factor 1 (SDF1)	
Mesenchymal Stromal	SCF	CXCL 12
	Stromal cell-derived factor 1	
Other Regulatory Cells		
Osteoblasts	N/A	CXCL 12 for lymphoid progenitors
Megakaryocytes	Transforming growth factor beta 1 (TGF- β 1)	CXCL 4
	Platelet factor 4	
Schwann (PNS)	N/A	TGF- β 1
Macrophages	N/A	IL1 (Interleukin1), TNF- α

neural Schwann cells, megakaryocytes, macrophages, and osteoblasts, influence the cell cycle, localization, and lineage of HSC through direct and indirect processes.¹⁸ Table 1-3 lists selected regulatory cells and the cytokines that support HSCs.

The hematopoietic system consists of the bone marrow, liver, spleen, lymph nodes, and thymus. These tissues and organs are involved in the production, maturation, and destruction of blood cells. The entire process of hematopoiesis evolves from the stem cells that support hematopoiesis, the progenitor cells that are committed to particular cell lines, and the regulatory factors (growth factors) to which the hematopoietic system responds. These features enable the hematopoietic system to respond to stimuli such as infection, bleeding, or hypoxia by increasing hematopoiesis, with an emphasis on the cell type needed.^{17,18}

Origin of Hematopoiesis

During the first few weeks of embryonic life, hematopoiesis begins in the mesoderm of the yolk sac (Fig. 1-16) with mesenchymal stem cells forming large primitive nucleated erythroid cells.¹³ Yolk sac production of these nucleated erythroid cells begins to decline in about 6 weeks and ends in about 2 months.¹⁹

The fetal liver assumes responsibility for hematopoiesis during the second month, with the yolk sac nucleated RBCs

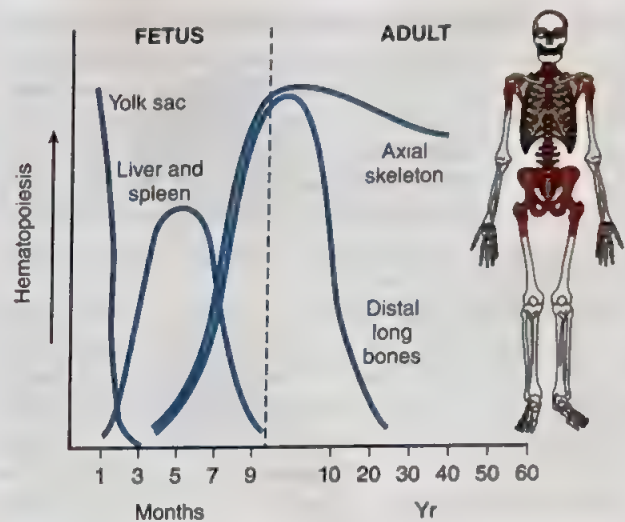


FIGURE 1-16 Location of active marrow growth in the fetus and adult. During fetal development, hematopoiesis is first established in the yolk sac mesenchyme, later moves to the liver and spleen, and finally is limited to the body skeleton. From infancy to adulthood, there is a progressive restriction of productive marrow to the axial skeleton and proximal ends of the long bones, shown as the shaded areas on the drawing of the skeleton.

migrating to the liver and remaining in the liver until the seventh month.¹⁹ From the third to the sixth month, splenic hematopoiesis also occurs. At approximately 7 months of fetal life, the responsibility for hematopoiesis shifts from the liver to the bone marrow, which then becomes the major site of blood cell development in the fetus.¹⁹ The fetal marrow becomes filled with RBCs during hematopoiesis. Bones of the toes, fingers, vertebrae, ribs, pelvis, long bones, and cranium are filled with erythroid cells; early lymphocytic cells also may be formed during fetal life. A few megakaryocytes (precursors to platelets) first appear at approximately 3 months of fetal life, and granulocytes are observed at about 5 months.¹⁹

At birth, the liver and spleen have ceased hematopoietic cell development, and the active sites of hematopoiesis are in bone cavities (red marrow). Bone seems to provide a microenvironment most appropriate for proliferation and maturation of blood cells.¹⁹ Hematopoiesis occurs in the extravascular part of the red marrow, with a single layer of epithelial cells separating the extravascular marrow compartment from the intravascular compartment (venous sinuses). When new blood cells produced in the marrow are almost mature and ready to circulate in the peripheral blood, the migrating cells leave the marrow parenchyma by squeezing through cytoplasmic fenestrations in sinus endothelial lining cells and emerging into venous sinuses.

During infancy and early childhood, hematopoiesis takes place in the entire medullary space, with the volume of marrow in the newborn infant almost equaling the hematopoietic marrow space of adults.¹⁹

Hematopoiesis gradually decreases in the shaft of the long bones, and after the age of 4 years, fat cells begin to appear in the long bones.¹⁹ Around age 18 to 20, hematopoietic marrow is present exclusively in the sternum, ribs, pelvis, vertebrae,

and skull.²⁰ Other bones contain primary fat (yellow marrow). After the age of 40, marrow in the sternum, ribs, pelvis, and vertebrae is composed of equal amounts of hematopoietic tissue and fat.²⁰ Generally, hematopoiesis is sustained in a steady state as production of mature cells equals blood cell removal. When there is increased demand for blood cells, active hematopoiesis may again occur in the spleen, liver, and other tissues as a compensatory mechanism known as *extramedullary hematopoiesis*.²⁰

Bone marrow hematopoietic activity can be divided into two separate pools—the stem cell pool and the bone marrow pool—with eventual release of mature cells into the peripheral blood (Fig. 1-17). It is assumed that in the bone marrow microenvironment there is a stem cell pool where morphologically unidentifiable **multipotent stem cells (MSCs)** and common multipotent and limited progenitor stem cells reside. The vast majority of HSCs in the marrow localize adjacent to blood vessels, therefore proximal to perivascular cells.¹⁵

There are also two separate granulocytic pools in the peripheral blood: those that are functional within the circulation and those that exist in a storage form. In the granulocytic cell line in the bone marrow pool, there is a component for proliferation and maturation, as well as a storage component.¹⁴ As seen in Figure 1-17, the granulocytic cells in the peripheral blood also contain 50% of circulating cells and 50% of storage cells.²⁰ The neutrophils that line the walls of the blood vessels are sometimes referred to as the *marginating storage pool*.¹⁴

For platelets (also known as thrombocytes), the peripheral blood contains 70% of platelets that circulate, with 30% being stored in the spleen.²⁰ Figure 1-17 demonstrates that the bone marrow pool consists of only proliferating and maturing platelet precursor cells.

One hundred percent of RBCs, known as erythrocytes, circulate in the peripheral blood in a functional state and in the bone marrow pool. Erythrocytes in various stages of development are a large component of proliferating and maturing red cell precursors found in the bone marrow. At birth, the

normal cellularity is 100%.²⁰ Afterward, the cellularity gradually decreases with age. Marrow cellularity in adults is approximately 50% ((10%).²¹ The general rule to estimate age-related normal ranges is 100 minus the age ± 10 .²¹ For example, the estimated normal marrow cellularity for a 50-year-old person would be $100 - 50 \pm 10$, representing a range from 40% to 60%. In the bone marrow, an average myeloid to erythroid ratio is 4:1 in terms of cellularity.²¹ Therefore in an adult with 50% marrow cellularity, approximately 40% represents granulopoiesis and 10% represents erythropoiesis.²² Table 1-4 lists the normal adult values for bone marrow cells

Erythropoiesis

The term *erythropoiesis* identifies the entire process by which erythrocytes are produced in the bone marrow. In response to EPO, a growth factor that stimulates the erythroid precursors, erythropoiesis occurs in the central sinus beds of medullary marrow over a period of about 5 days through at least three successive reduction-divisions from pronormoblast to basophilic normoblast to polychromatophilic normoblast and finally to orthochromatic normoblast.²³ With successive developmental stages, the following changes occur: reduction in cell volume, condensation of chromatin, decrease in N:C ratio, loss of nucleoli, decrease in ribonucleic acid (RNA) in the cytoplasm, decrease in mitochondria, and gradual increase in synthesis of hemoglobin (Fig. 1-18).

It is helpful to memorize the following developmental stages from the “mother cell” to mature erythrocyte: **pronormoblast** (rubriblast) to **basophilic normoblast** (prorubricyte) to **polychromatophilic normoblast** (rubricyte) to **orthochromatic normoblast** (metarubricyte). The nucleus of the orthochromatic normoblast is eventually extruded, leaving a nonnucleated polychromatophilic (diffusely basophilic) erythrocyte (**reticulocyte**), which is released into the circulating blood to mature in 1 to 2 days.²⁴ Progressive cellular divisions of one pronormoblast results in production of 14 to 16 erythrocytes.²⁴

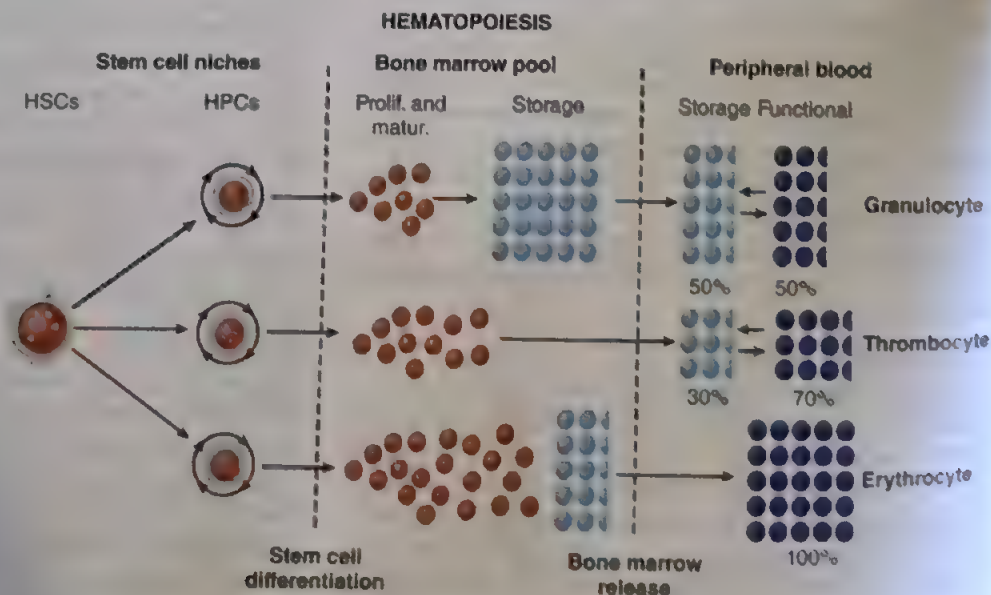


FIGURE 1-17 Hematopoiesis.

TABLE 1-4 Bone Marrow Cells: Normal Adult Values

Cell	Percentage
Stem cell	0–0.01
Myeloblast	0–2
Promyelocyte	1–4
N. myelocyte	5–20
N. metamyelocyte	5–20
N. band	10–35
N. segmented	5–15
Eosinophil	0–3
Basophil	0–1
Lymphocyte	5–15
Plasmacyte	0–1
Monocyte	0–2
Other cells	0–1
Megakaryocyte	0.1–0.5
Pronormoblast	0–1.5
Basophilic normoblast	1–5
Polychromatophilic normoblast	5–30
Orthochromatic normoblast	5–10
Myeloid to Erythroid Ratio (M:E) = 4:1	

Source: Percentage of total nucleated cells in bone marrow represent normal reference ranges for adults taken from the University of Texas Health Science Center and University Hospital, San Antonio, Texas.

Pronormoblast (Rubriblast, Proerythroblast)

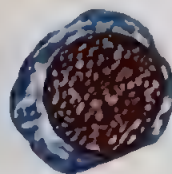
The pronormoblast, the earliest recognizable cell of the erythrocytic series, has a round, primitive nucleus with visible nucleoli and chromatin strands that are distinct and dispersed. There is no evidence of clumped chromatin. The nucleus stains reddish-blue with Wright's stain. The cytoplasm stains a deep blue owing to the presence of RNA.³ The nuclear-to-cytoplasmic (N:C) ratio in a pronormoblast is 8:1 to 6:1 (Figs. 1-19A, 1-20A, 1-21, 1-22, and 1-23A).²⁴

Pronormoblasts range in size between 14 and 24 μm .²⁰ A pronormoblast is usually slightly larger than a myeloblast and has more cytoplasm, which stains a deeper blue. Pronormoblasts constitute 1.5% or less of the cells observed in normal bone marrow (see Table 1-4). Pronormoblasts usually divide within 12 hours to make daughter cells (basophilic normoblasts).²⁴ The morphological characteristics of the erythrocytic series are summarized and illustrated in Table 1-5.

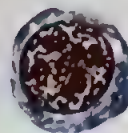
Basophilic Normoblast (Prorubricyte, Basophilic Erythroblast)

Basophilic normoblasts, the daughter cells of pronormoblasts, require about 20 hours to develop. In normal bone marrow there are about four times as many basophilic normoblasts as pronormoblasts.²⁴ The basophilic normoblast is differentiated

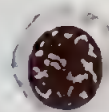
NORMAL ERYTHROCYTIC SEQUENCE



Pronormoblast



Basophilic normoblast



Polychromatophilic normoblast



Orthochromatic normoblast



Polychromatophilic erythrocyte (reticulocyte)



Mature erythrocyte

FIGURE 1-18 Erythrocytic system. Erythropoiesis. (From Diggs LW, et al. *The Morphology of Human Blood Cells*, ed 5, 1985, pp 1-18, 25-27. Abbott Laboratories, Abbott Park, IL, with permission.)

from the pronormoblast by the coarsening of the chromatin pattern and the nucleoli, which are ill-defined or not visible under light microscopy. As the basophilic normoblast matures, it accumulates more RNA and hemoglobin (Figs. 1-24, 1-25A, 1-26, and 1-27). The predominant color of the cytoplasm is blue due to the staining of RNA, but there may be a pinkish tinge reflecting the presence of varying amounts of hemoglobin. The N:C ratio in the basophilic normoblast is 6:1 to 4:1.²⁴ A basophilic normoblast is somewhat smaller than a pronormoblast, with a size of 12 to 17 μm .²⁴ Normal bone marrow contains 1% to 5% basophilic normoblasts (see Table 1-4). The division of the basophilic normoblasts forms polychromatophilic normoblasts, which are smaller than basophilic normoblasts but have twice the amount of hemoglobin.

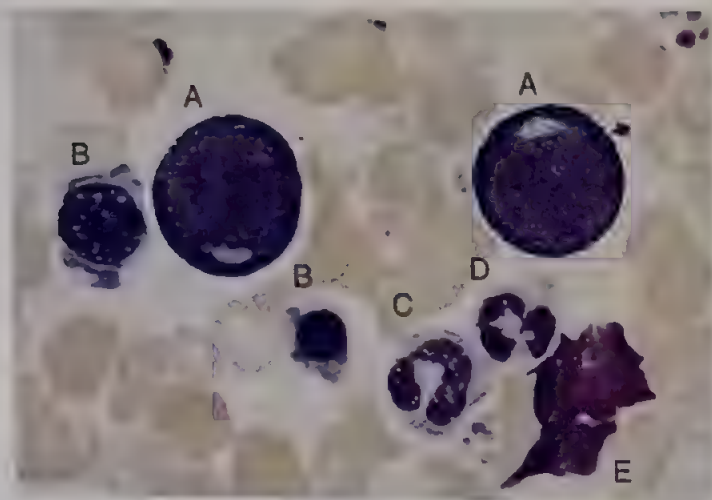


FIGURE 1-19 A. Two pronormoblasts (note the perinuclear halo). B. One polychromatophilic normoblasts (left); one orthochromatic normoblast (below center). C. Neutrophilic band. D. Segmented neutrophil. E. Smudge cell.

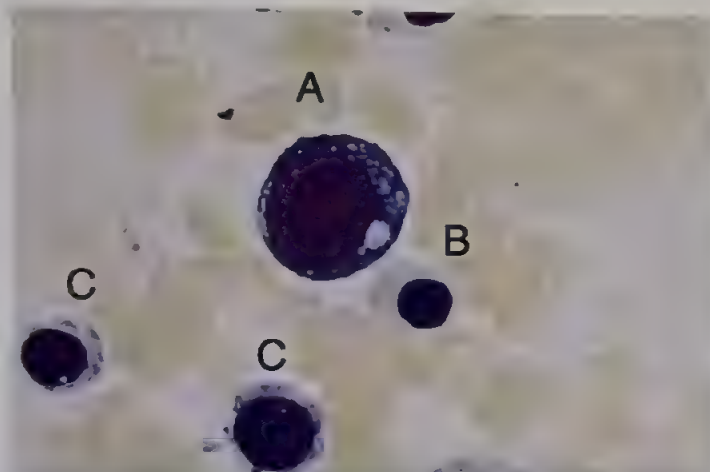


FIGURE 1-20 A. Pronormoblast. B. Orthochromatic normoblast. C. Two polychromatophilic normoblasts.

Polychromatophilic Normoblast (Rubricyte, Polychromatophilic Erythroblast)

Polychromatophilic normoblasts are smaller than basophilic normoblasts (10 to 15 μm), having relatively more cytoplasm and a smaller nucleus than basophilic normoblasts.²⁴ The cytoplasm contains a varying mixture of pink due to hemoglobin and blue due to RNA; in the late polychromatophilic normoblast, the pinkish color is usually predominant.

Nuclear chromatin is thickened and irregularly condensed in the polychromatophilic normoblast. Light-staining parachromatin areas are visible among the dark blue-staining, irregular pyknotic masses. Nucleoli are no longer visible. The N:C ratio in a polychromatophilic normoblast is 4:1 to 2:1²⁴ (see Figs. 1-18, 1-19B, 1-20C, 1-25B, 1-28, and 1-29A).

The maturation time for polychromatophilic normoblasts in bone marrow is about 30 hours, and there are approximately three times as many polychromatophilic normoblasts as basophilic normoblasts in the bone marrow. Bone marrow

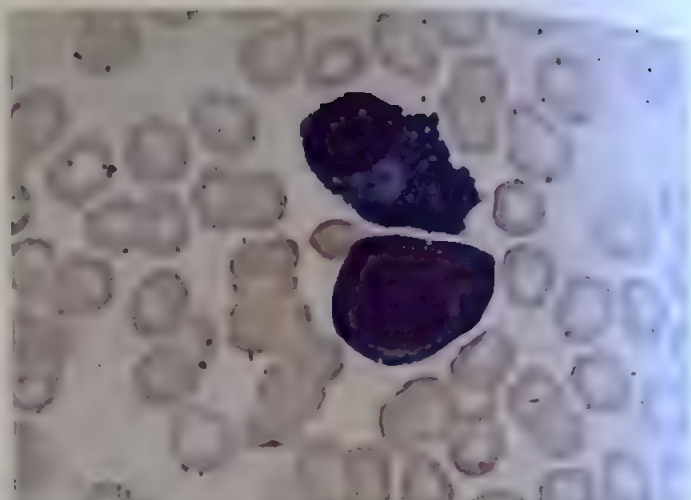


FIGURE 1-21 Center: Pronormoblasts; upper center: Plasmacyte.

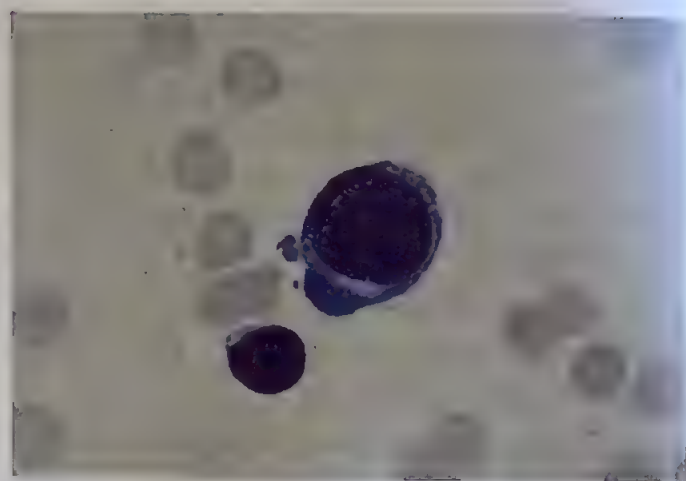


FIGURE 1-22 Center: Pronormoblasts; lower center: Lymphocyte.

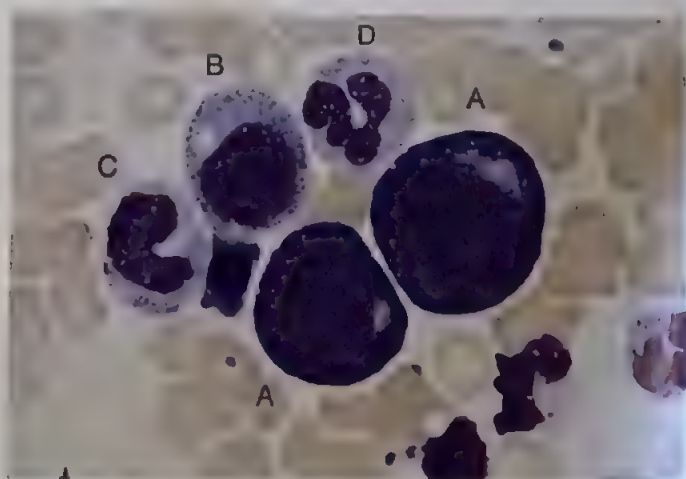

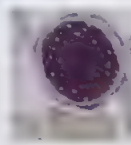






FIGURE 1-23 A. Pronormoblasts. B. Neutrophilic myelocyte. C. Neutrophilic metamyelocyte. D. Segmented neutrophil.

in a normal adult contains 5% to 30% polychromatophilic normoblasts. Polychromatophilic normoblasts are *not* present in the normal peripheral blood of adults, but they may appear in small numbers in the peripheral blood of normal newborn infants.¹⁹

TABLE 1-5 Morphological Characteristics of the Erythrocytic Series

Name	Cell	% in Blood Marrow	Cell Size, μm	N:C Ratio	Nuclear Shape	Nuclear Position	Nuclear Color/Chromatin	Nucleoli	Color/Amount of Cytoplasm	Cytoplasmic Granules
Pronormoblast (rubriblast)		0–1.5%	14–24	8:1–6:1	Round	Central	Reddish-blue finely stippled granular chromatin	0–2	Dark or royal blue/slight	None
Basophilic normoblast (prorubricyte)		1–5%	12–17	6:1–4:1	Round	Central	Increased, larger granularity of nuclear chromatin	Usually none, occasional indistinct nucleolus	Basophilic/slight	None
Polychromatophilic normoblast (rubricyte)		5–30%	10–15	4:1–2:1	Round	Central	Dark blue, smaller nucleus with parachromatin, increased clumped chromatin	None	Bluish-pink/moderate	None
Orthochromatic normoblast (metarubricyte)		5–10%	8–12	1:1–1:2	Round	Central	Blue-purple, small nucleus with pyknotic degeneration/condensed chromatin	None	Pink/moderate	None
Polychromatophilic erythrocyte (reticulocyte)		n/a	7–10	n/a	n/a: nucleus has been extruded	n/a	n/a	None	Clear gray-blue, polychromatophilic to pink	None
Mature erythrocyte		n/a	7–8	n/a	n/a	n/a	n/a	None	Pink	None

BM = bone marrow; n/a = not applicable % in BM = Percentage of total nucleated cells in bone marrow represent normal reference ranges for adults taken from the University of Texas Health Science Center and University Hospital, San Antonio, Texas.

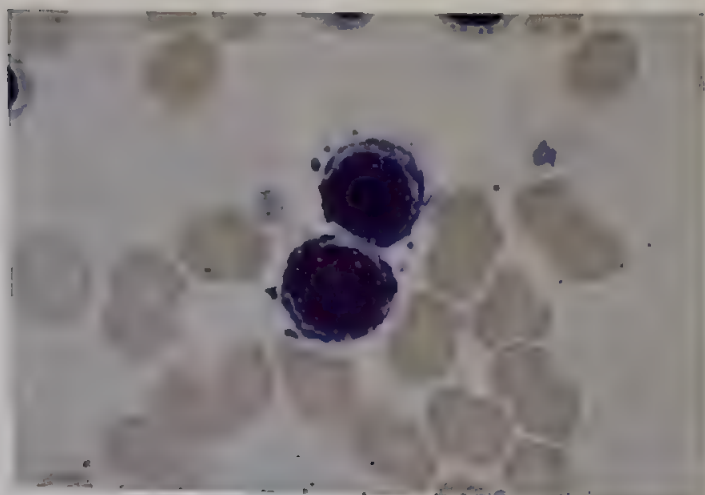


FIGURE 1-24 Basophilic normoblasts.

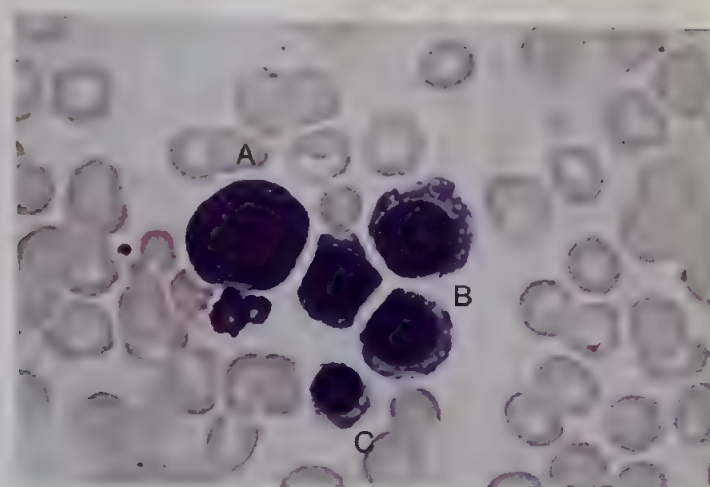


FIGURE 1-25 A. Basophilic normoblast. B. Three polychromatophilic normoblasts. C. Orthochromatophilic normoblast.

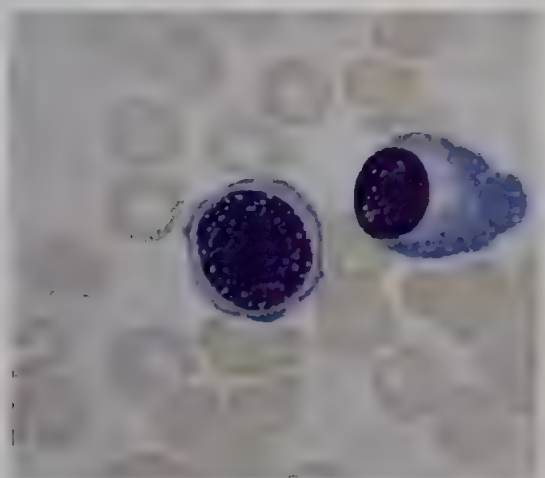


FIGURE 1-26 Left: Basophilic normoblast; center: Plasmacyte.

Orthochromatophilic Normoblast (Metarubricyte, Orthochromatophilic Erythroblast)

Orthochromatophilic normoblasts are formed from polychromatophilic normoblasts and are recognized by the solid, blue-black, degenerated nucleus with a nonlinear clumped chromatin pattern (see Figs. 1-18, 1-20B, 1-25C, and 1-27). The nucleus is called pyknotic because there is no parachromatin (white areas) present. The orthochromatophilic normoblast

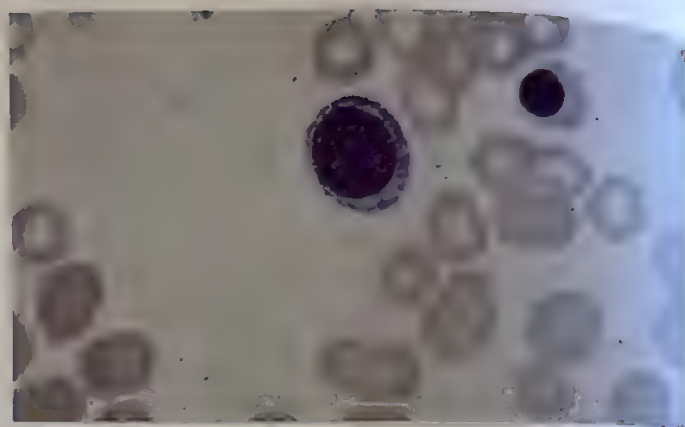


FIGURE 1-27 Center: Basophilic normoblast; right: Orthochromatophilic normoblast.



FIGURE 1-28 Polychromatophilic normoblasts: early and late stages.

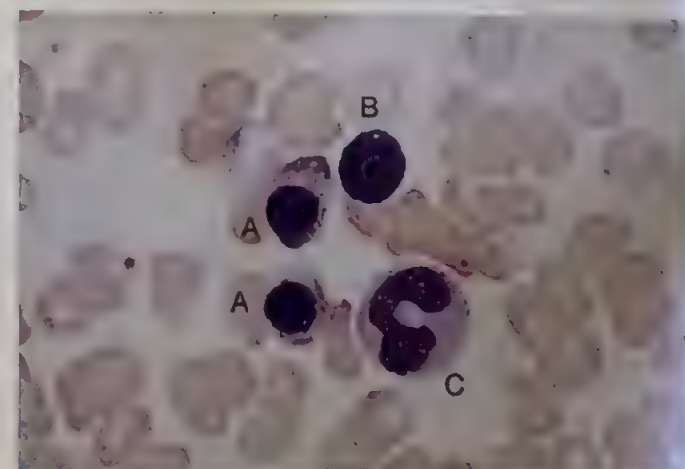


FIGURE 1-29 A. Orthochromatophilic normoblasts. B. Lymphocyte. C. Segmented neutrophil.

nucleus is incapable of further DNA synthesis and therefore cannot divide. The degenerated nucleus of the orthochromatophilic normoblast is destined to be extruded and will be phagocytized. The N:C ratio in an orthochromatophilic normoblast is 1:1 to 1:2.²⁴

The cytoplasm is predominantly pink (or reddish) because of increasing hemoglobin synthesis, but there may remain minimal amounts of blue cytoplasm due to the presence of RNA.

The maturation time for the orthochromatic normoblast is 48 hours. The number of orthochromatic normoblasts in normal bone marrow varies between 5% and 10% (see Table 1-4). Orthochromatic normoblasts are not observed in the normal peripheral blood of adults, but they can be found in the blood of normal newborn infants.¹⁹ The orthochromatic normoblast is the smallest of the nucleated erythrocyte precursors (8 to 12 μm). Morphological characteristics of orthochromatic normoblasts are given in Table 1-5.

Reticulocyte (Diffusely Basophilic Erythrocyte, Polychromatophilic Erythrocyte)

The condensed, pyknotic nucleus of an orthochromatic normoblast is extruded, leaving a diffusely basophilic or polychromatophilic cell. The membrane of the erythrocyte seals itself. Some of the bluish-staining color remains because of the presence of RNA. The erythrocyte contains approximately two-thirds of its total hemoglobin content by the time the nucleus is lost. The RNA content soon begins to decrease.

A diffusely basophilic erythrocyte is larger than a mature red cell (8 to 10 μm).²⁴ It is released in 2 to 3 days from the marrow and circulates for 1 or 2 days before maturing into an erythrocyte.²⁴ Only rarely are diffusely basophilic erythrocytes found in the blood of normal adults; however, polychromatophilic cells are frequently seen in the blood of normal newborn infants.¹⁹

When stained with new methylene blue, these polychromatophilic erythrocytes reveal ribosomes in a **granulofilamentous** arrangement (or network of strands and granules) and are classified as reticulocytes (Fig. 1-30). As ribosomes disappear, the diffusely basophilic cell changes into a mature erythrocyte.

With anemia or hypoxia, EPO stimulates marrow erythroid precursors to proliferate and to increase the number of early erythroid cells. An increased number of polychromatophilic cells are delivered early from the marrow and, therefore, the reticulocyte count is increased.²⁰



FIGURE 1-30 Reticulocytes. New methylene blue stain of peripheral blood. Note reticulocytes with varying amounts of stained reticulum (RNA). Reticulocytosis is associated with increased erythropoietic activity reflected by polychromasia on the Wright's stain of the peripheral blood.

Erythrocyte (Red Blood Cell, Discocyte)

The morphological characteristics of a normal erythrocyte were presented at the beginning of this chapter.

A mature erythrocyte is not able to synthesize hemoglobin, because it is without a nucleus, mitochondria, or ribosomes, but it has a unique, yet limited, metabolism to sustain itself while traversing the microvasculature. The erythrocyte carries oxygen from the lungs to the tissues where it is exchanged for carbon dioxide. Erythrocytes are pliable or flexible and deformable, making them capable of unusual changes in shape that are necessary for the passage through the microcirculation to transport oxygen. Refer to Table 1-5 for a summary of the morphological characteristics of each stage of maturation of the red cell.

Myelopoiesis (Granulocytopoiesis)

Myelopoiesis (granulocytopoiesis) refers to the production of neutrophils, eosinophils, and basophils (Fig. 1-31). Mature neutrophils, eosinophils, and basophils have similar patterns of proliferation, differentiation, division, storage in marrow, and delivery to the blood. Maturation and division of the myeloid series in the marrow demonstrate a continuum of development from the blast to the most mature cell (segmented neutrophil), requiring from 7 to 11 days.²⁴

Granulocyte production proceeds after the cell lineage commitment has determined the identity of the maturing cell as a member of the myelocytic series. These cells actively divide and mature, taking 1 to 2 days for each cellular cycle. The maturation pool is composed of metamyelocytes and bands, and represents the end of DNA synthesis and division. The transformation of myelocyte to metamyelocyte to band takes about 8 to 9 hours after entry into the maturation pool.²⁴ The storage pool retains mature cells for release into peripheral circulation. These mature cells leave the marrow by moving through transiently formed pores in endothelial cells that separate marrow parenchyma from venous sinuses. When leaving blood for tissue, these cells migrate between endothelial cells (**diapedesis**). After release, these cells become part of the functional pool and reside as circulating cells or as marginated cells, which line blood vessel walls. Cells are released to enter the peripheral blood or vessel walls for a few hours and then leave the blood to enter the tissues and body cavities. As these cells exit the blood or the tissues, they are replaced by other cells from the marrow. Once in the blood, half of the released cells freely circulate while the other half are in a **marginating neutrophil pool** on the walls of blood vessels, particularly those in lungs, liver, and spleen. These latter cells leave the peripheral vessel to be directed by chemotactic factors to inflammatory or infectious tissue. After these cells enter tissues, they do not reenter the circulation or the marrow.²⁴

Morphological Changes

Many morphological changes occur during maturation of granulocytes. These include a reduction in nuclear volume, condensation of chromatin, change in nuclear shape, appearance and disappearance of primary granules, appearance of

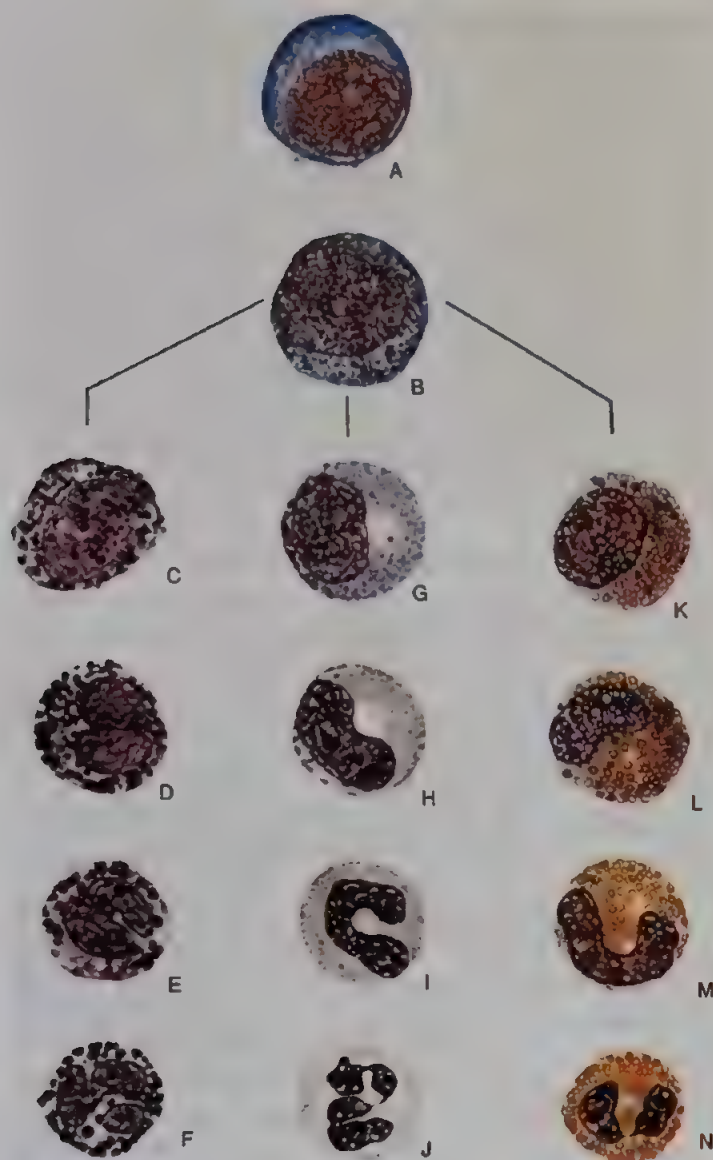


FIGURE 1-31 Granulocytopoiesis: myelocytic (granulocytic) system. A. Myeloblast. B. Promyelocyte (progranulocyte). C. Basophilic myelocyte. D. Basophilic metamyelocyte. E. Basophilic band. F. Segmented basophil. G. Neutrophilic myelocyte. H. Neutrophilic metamyelocyte. I. Neutrophilic band. J. Segmented neutrophil. K. Eosinophilic myelocyte. L. Eosinophilic metamyelocyte. M. Eosinophilic band. N. Segmented eosinophil. (From Diggs LW, et al. *The Morphology of Human Blood Cells*, ed 5, 1985, pp 1-18, 25-27. Abbott Laboratories, Abbott Park, IL, with permission.)

secondary granules, color changes in cytoplasm from blue to pinkish-red, and change in the size of cells³ (Table 1-6).

Maturation of the granulocytic series of cells is characterized by the development of primary blue-staining granules, which are replaced by secondary granules that differ in their affinity for various dyes. Cells with an affinity for basic dyes are basophils; the cells that stain reddish-orange with the acid dye eosin are eosinophils; the cells that do not stain intensely with either acid or basic dyes are called neutrophils. As these motile cells mature, the nucleus undergoes progressive changes from round to multilobular forms.

Stages of Differentiation and Maturation

Myeloblasts

The earliest recognizable cell in the granulocytic series is a myeloblast.²⁵ A myeloblast usually has a round nucleus that

stains predominantly reddish-blue and has a smooth nuclear membrane. The interlaced chromatin strands are delicate, finely dispersed or stippled, and evenly stained but not clumped. One or more nucleoli of uniform size are usually demonstrable, but occasionally nucleoli may be barely visible. A slight to moderate amount of bluish nongranular cytoplasm stains lighter next to the nucleus than at the periphery of the cell (see Figs. 1-31A and 1-32). The N:C ratio in the myeloblast is 7:1 to 5:1.²⁴ A myeloblast is smaller and has less blue cytoplasm than a pronormoblast. After about three to five mitotic divisions, the myeloblast matures into a promyelocyte as primary granules become visible.

Myeloblasts vary in size from 15 to 20 μm .²⁴ They are not present in normal peripheral blood. Normal marrow contains 2% or less myeloblasts. The appearance of primary granules marks the maturation of the myeloblast into a promyelocyte.

Promyelocyte (Progranulocyte)


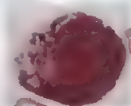




The promyelocyte contains granules that stain dark blue or reddish-blue and may be round or irregular in shape. They appear scattered throughout the cytoplasm and may overlay the nucleus.

The nucleus of a promyelocyte is usually round and large in relation to the cytoplasm. The chromatin of young promyelocytes is almost as finely granular as it is in a myeloblast. In older cells, the chromatin structure is slightly coarser than that in a myeloblast. Nucleoli may be faintly visible but are not often distinct (see Figs. 1-31B, 1-33, and 1-34).³ The N:C ratio in a promyelocyte is 5:1 to 3:1.²⁴ The cytoplasm is blue, with a relatively light zone adjacent to the nucleus. The periphery of the cytoplasm is smooth and not indented by neighboring cells. The size of a promyelocyte may vary from 12 to 24 μm , depending on the stage of a given cell in the mitotic cycle. It is often 20 μm and may be larger than a myeloblast.³ Promyelocytes are not present in normal peripheral blood. From 1% to 4% promyelocytes are observed in normal bone marrow.²⁴ As the promyelocyte matures, nucleoli begin to fade, the chromatin becomes more condensed, and the granules are not as intensely stained. Specific secondary neutrophilic granules begin to appear, and the synthesis of primary granules ceases. A few primary granules remain through division and maturation, and may even appear in segmented neutrophils.

Neutrophilic Myelocytes

When primary granules are no longer synthesized and less dense secondary neutrophilic granules can be identified, the cell has matured into a myelocyte. The first sign of neutrophilic differentiation has been called the "dawn of neutrophilia" or "beginning neutrophilia," which refers to a relatively light island of ill-defined or barely visible (pinkish) secondary lysosomal granules that develop adjacent to the nucleus and in proximity to the remaining primary granules. As myelocytes divide and age, the primary granules become fewer and the secondary (specific) neutrophilic granules predominate.²⁴ Secondary granules are considered to be specific granules for neutrophils, and they contain collagenase, lysozyme, lactoferrin, plasminogen activators, and aminopeptidase.²⁰

TABLE 1-6 Morphological Characteristics of the Granulocytic (Neutrophilic) Series

Name	Cell	% in BM	Cell Size, μm	N:C Ratio	Nuclear Shape	Nuclear Position	Nuclear Color/ Chromatin	Nucleoli	Color/Amount of Cytoplasm	Cytoplasmic Granules
Myeloblast		0–2%	15–20	7:1–5:1	Round to oval	Eccentric or central	Light reddish-blue, fine meshwork, with no aggregation of material	1–3	Basophilic/ slight	Absent
Promyelocyte		1–4%	12–24	5:1–3:1	Round to oval	Eccentric or central	Light reddish-blue, fine meshwork, slight aggregation may be seen at nuclear membrane	1–2	Basophilic/ increased	Present, fine azurophilic, nonspecific granules
Neutrophilic myelocyte		5–20%	10–18	2:1–1:1	Oval or round: slightly indented	Usually eccentric	Reddish-blue fine chromatin with slightly aggregated or granular pattern	May or may not have nucleolus	Bluish-pink/ moderate	Present, azurophilic, specific granules
Neutrophilic metamyelocyte		5–15%	10–18	1:1	Usually indented (kidney-shaped)	Central or eccentric	Light blue-purple with basophilic chromatin easily distinguishable	None	Clear pink/ moderate	Present, (specific) granules, neutrophilic
Neutrophilic band		10–35%	10–16	1:1–1:2	Elongated, narrow band (horseshoe) shape of uniform thickness	Central or eccentric	Purplish-red, clumped granular chromatin	None	Pink/ abundant	Specific granules, fine violet-pink
Neutrophilic segmented (neutrophils or polys)		5–15%	10–16	1:3	2–5 distinct nuclear lobes	Central or eccentric	Purplish-red clumped granular chromatin	None	Pink/ abundant	Specific granules, fine violet-pink

% in BM = Percentage of total nucleated cells in bone marrow represent normal reference ranges for adults taken from the University of Texas Health Science Center and University Hospital, San Antonio, Texas.

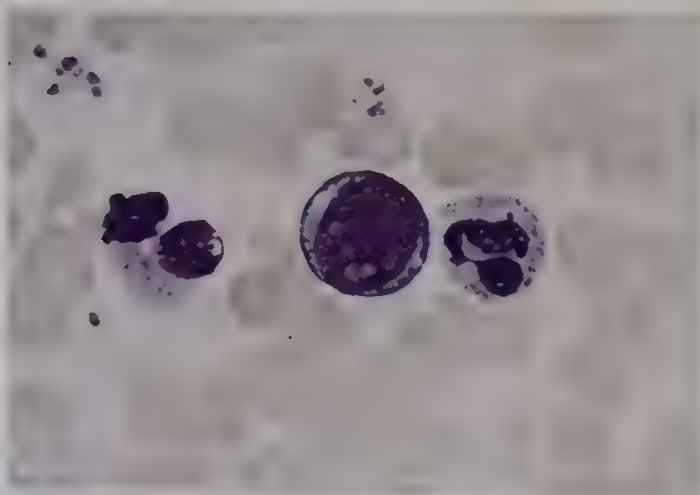


FIGURE 1-32 Center: Myeloblast; right: Segmented neutrophil; left: disintegrated neutrophil.

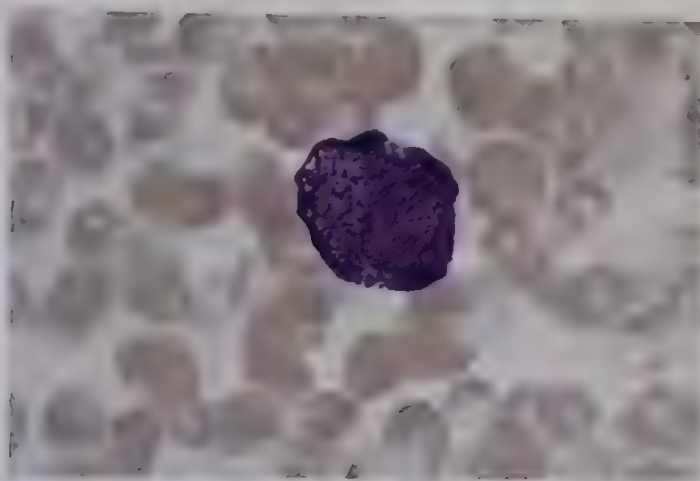


FIGURE 1-33 Promyelocyte.

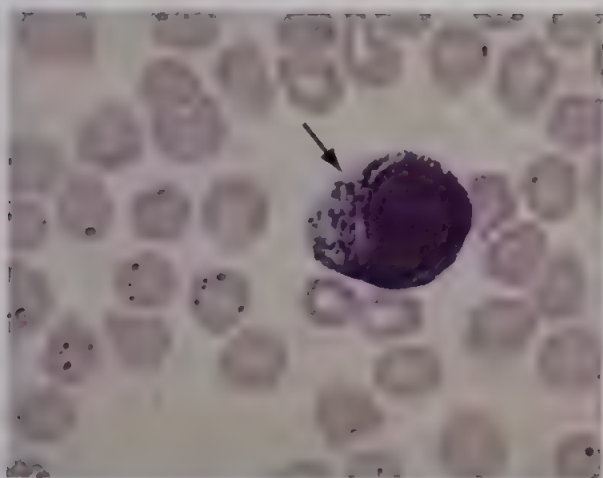


FIGURE 1-34 Promyelocyte.

The nuclei of myelocytes may be round, oval, or flattened on one side and usually decentralized³ (see Figs. 1-31G and 1-35A). Chromatin strands become condensed, partly clumped, and thickened, and are unevenly stained. Nucleoli are absent or indistinct in myelocytes. The neutrophilic myelocyte is the last myeloid precursor capable of division.³

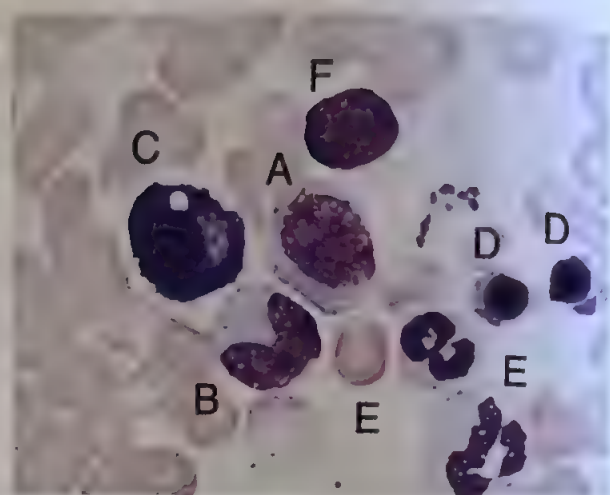


FIGURE 1-35 A. Neutrophilic myelocyte. B. Neutrophilic metamyelocyte. C. Plasmacyte. D. Orthochromatic normoblasts. E. Segmented neutrophils. F. Nucleus of a degenerated cell.

Neutrophilic myelocytes are often smaller than promyelocytes (10 to 18 μm) and have relatively large amounts of cytoplasm (N:C ratio is 2:1 to 1:1),³ which gradually becomes less basophilic and more pinkish. The normal peripheral blood does not contain neutrophilic myelocytes. There are 5% to 20% myelocytes in normal bone marrow.²⁴

Neutrophilic Metamyelocytes

As maturation proceeds, the nucleus becomes slightly indented (bean- or kidney-shaped), and this shape serves to identify the cell as a metamyelocyte. The indentation is less than half the width of an arbitrary round nucleus (Fig. 1-36).³ There is noticeable condensation with clumping of the chromatin, but the chromatin structure is not as dense as that of the segmented neutrophilic cell. Metamyelocytes do not divide nor do they have nucleoli.¹⁴ The N:C ratio is 1:1.³

Many small, pinkish secondary granules fill the cytoplasm, and there may be a few primary darker granules remaining (see Figs. 1-31H, 1-35B, and 1-37A). These maturing cells remain in the bone marrow and represent a portion of the granulocytic reserve.²⁴

Metamyelocytes are somewhat smaller than myelocytes (10 to 18 μm) and are larger than the band neutrophil or segmented cell.²⁴ These cells are usually absent in normal peripheral blood. There are approximately 5% to 20% metamyelocytes in normal bone marrow.²⁴

Band Neutrophil

When the stage is reached in which the nuclear indentation in the early granulocyte is greater than half the width of

TERMINOLOGY BASED ON INDENTATION OF NUCLEI

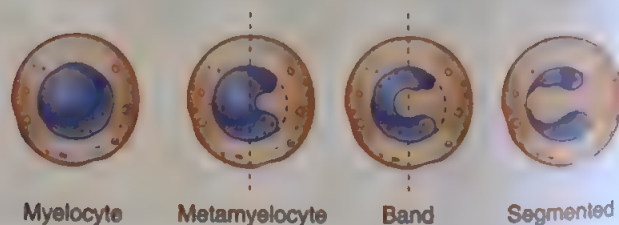


FIGURE 1-36 Terminology based on indentation of nuclei: (left to right) myelocyte, metamyelocyte, band, segmented.

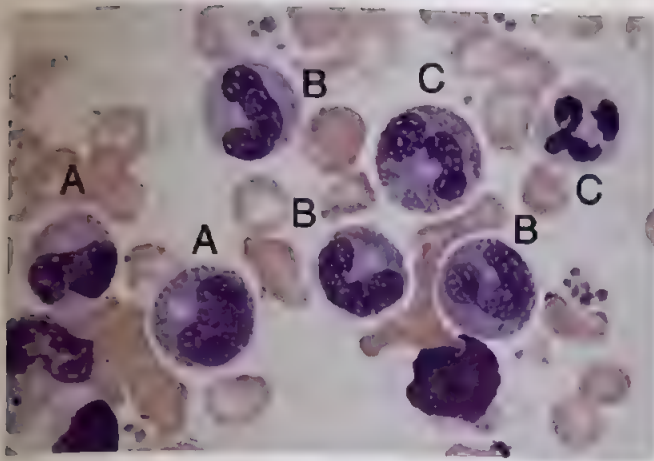


FIGURE 1-37 A. Two neutrophilic metamyelocytes. B. Three neutrophilic bands. C. Two segmented neutrophils.

the nucleus (see Fig. 1-36), the cell is identified as a band neutrophil.³

The opposite edges of the nucleus become almost parallel, giving the appearance of a horseshoe, hot dog, or a curved link of sausage. The shape of the nucleus of a band neutrophil is often folded or twisted, giving rise to difficulty in distinguishing a band from a segmented neutrophil. The nuclear chromatin is pyknotic, and there is usually a dark condensed mass at each end where the lobe is destined to be. The small secondary neutrophilic granules are evenly distributed and stain various shades of pink (see Figs. 1-31I and 1-37B). An occasional dark primary granule may be observed. The N:C ratio of a neutrophilic band is 1:1 to 1:2.²⁴

Neutrophilic band cells are often slightly smaller than metamyelocytes. Band forms constitute from 10% to 35% of the nucleated cells in the bone marrow.²⁴

Segmented neutrophils as stated earlier in this chapter, the nucleus of the segmented neutrophil is divided into two to five (often three) lobes that are connected by a thin filament or strand (see Figs. 1-31J, 1-32, 1-35E, 1-36, and 1-37C). The N:C ratio of a segmented neutrophil is 1:3.²⁴ Approximately 5% to 15% segmented neutrophils are noted in normal bone marrow of older children and adults.²⁴ In circulation, neutrophils have a short life span of an average of 9 hours, which demands constant production and release from the BM.²²

Neutrophils are now recognized as important regulators of the hematopoietic niche and, ultimately, of the stem cells from which they are derived.¹⁵

Because there is a gradual transition between the various stages of granulocytes, the division of neutrophils into developmental stages is somewhat arbitrary. This division is, however, necessary for morphological evaluation. Borderline cells that are difficult to distinguish from each other are often present. In this dilemma, the borderline cell should be classified as the more mature cell.²² See Table 1-6 for the morphological characteristics of the granulocytic (neutrophilic) series.

Tissue Neutrophils

Tissue neutrophils are large marrow cells with ample cytoplasm having irregular, blunt pseudopods that are often multipointed and may have nebulous cytoplasmic streamers (Fig. 1-38).

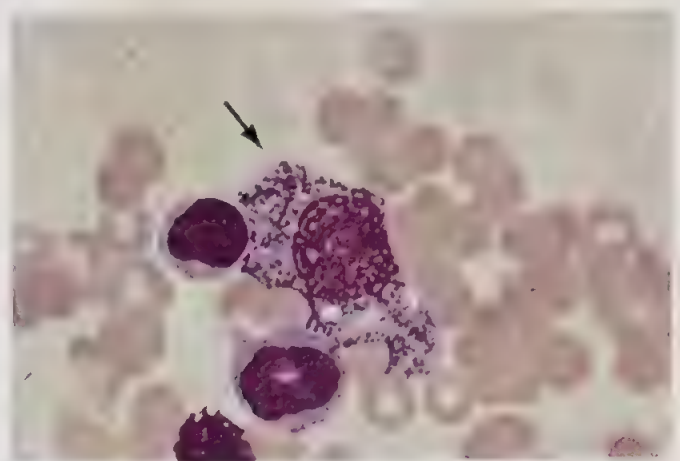


FIGURE 1-38 Tissue neutrophil (large center cell).

Eosinophils

Eosinophils pass through the same developmental stages as neutrophils: myelocyte, metamyelocyte, band, and segmented stages (see Figs. 1-31K through N). The earliest eosinophil (eosinophilic myelocyte) has a few dark bluish primary granules intermingled with the few specific, reddish-orange granules (Fig. 1-39). During development, the bluish granules become less visible and disappear, and the very round, specific, or secondary bright red refractile eosinophilic granules fill the cytoplasm (Figs. 1-40A, 1-41A, and 1-42A).

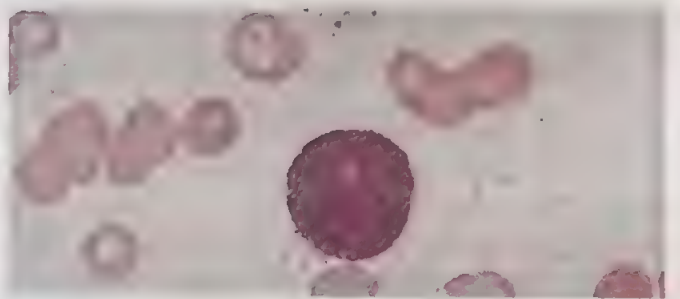


FIGURE 1-39 Center: Eosinophilic myelocyte.

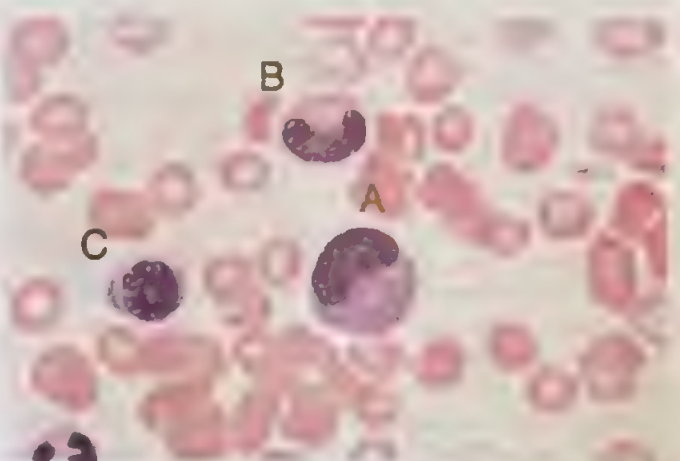


FIGURE 1-40 A. Eosinophilic metamyelocyte. B. Neutrophilic band. C. Polychromatophilic normoblast.

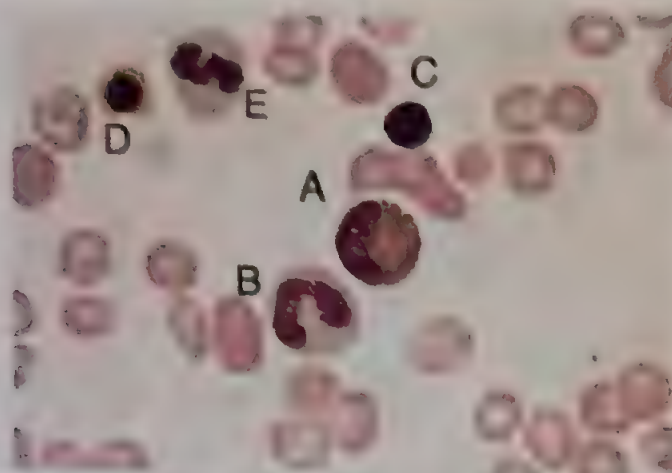


FIGURE 1-41 A. Eosinophilic band. B. Neutrophilic band. C. Lymphocyte. D. NRBC (orthochromatic normoblast). E. Segmented neutrophil.

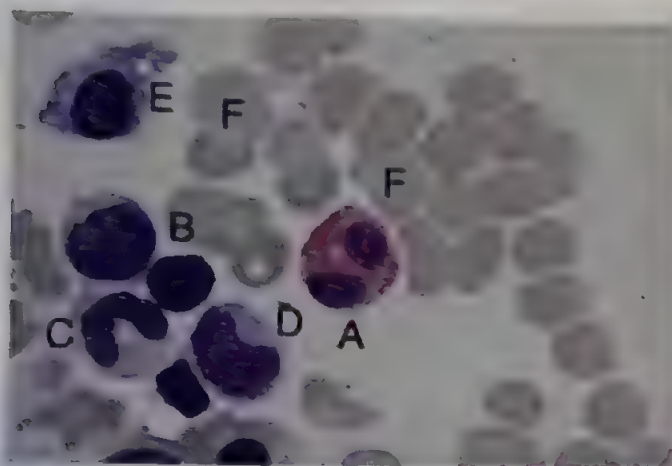


FIGURE 1-42 A. Segmented eosinophil. B. Lymphocyte. C. Neutrophilic band. D. Neutrophilic metamyelocyte. E. Plasmacyte. F. Two diffusely basophilic red cells.

Production of eosinophils in the marrow takes 3 to 6 days before the eosinophils appear in the peripheral blood.²⁴ Bone marrow provides a storage area for eosinophils so that they can be rapidly mobilized when needed. The factors that regulate production and release of eosinophils into blood are probably different from those that regulate neutrophils. The mean transit time of these cells in the circulatory system of humans has been reported to be about 8 hours, but in some disease states with eosinophilia, the time may be longer.²⁶ Much less is known about the stem cell kinetics of the eosinophil than of the neutrophil.

Eosinophils migrate from blood to tissue, such as bronchial mucosa, skin, gastrointestinal tract, and vagina in about 12 days.²⁷ Eosinophils may migrate from tissue back into blood and marrow. Eosinophils, which are motile, can migrate between endothelial cells into the tissue or into an area of inflammation in the same manner as neutrophils.²⁸

Normal adult bone marrow contains 0% to 3% eosinophils.²⁴ The morphological characteristics of the granulocytic (eosinophilic) series are summarized and illustrated in Table 1-7.

Tissue Eosinophils

In smears of bone marrow, occasionally there may be a large cell with elongated and tapering cytoplasmic extensions containing typical reddish-orange granules of the type seen in the eosinophils of the circulating blood²⁴ (Fig. 1-43).

Basophils. Basophils may be identified as basophilic myelocytes, metamyelocytes, bands, and segmented cells based upon the shape of their nuclei. The shape of the nucleus is, however, often masked by large basophilic granules (see Figs. 1-31C through F). The specific violet-blue granules of basophils are formed in the myelocytic stage and continue to be produced throughout all later maturation stages (see Figs. 1-31C through F).

Maturation of basophils in the bone marrow takes place over 7 days.²⁴ Mature basophils rarely have more than two segments. Basophils circulate for a few hours in blood, then migrate into skin, mucosa, and other serous membranes.²⁰

Basophils in all stages of maturation are smaller than promyelocytes and neutrophil myelocytes; their size approximates that of neutrophils. The morphological characteristics of the basophilic series are summarized and illustrated in Table 1-8. Normal bone marrow has 0% to 1% basophils.²⁴

Tissue Basophils (Mast Cells)

Tissue basophils (mast cells) (Fig. 1-44) and blood basophils are closely related in their functions and biochemical characteristics, but the relationship between them is still being studied. Tissue basophils are widely scattered in the connective tissue of various organs, bone marrow, and the mucosal area of serous membranes.²⁰

CRITICAL THINKING QUESTION

1-2 Differentiation of the different maturation stages of granulocytes can be difficult. What characteristics can morphologists utilize to help identify the correct maturation stage?

Monopoiesis


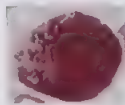




The mononuclear phagocyte system (MPS) is composed of monocytes, macrophages, and their precursors—monoblasts and promonocytes. The cells composing this system arise in the bone marrow from progenitor cells that are committed to monocyte-macrophage production.²⁷

Monoblasts and Promonocytes

Monoblasts are large and have an eccentrically placed nucleus that may be minimally indented; one or two large prominent nucleoli; a fine, lacy nuclear chromatin; and a nongranular cytoplasm that stains a deep blue (Fig. 1-45B). The N:C ratio in these cells is 7:1 to 4:1 (Table 1-9).²⁴ Monoblasts are nonmotile and nonphagocytic cells. Monoblasts divide and give rise to promonocytes and then to monocytes.

Promonocytes also are large and have indented or folded nuclei and fine chromatin. They often have a visible nucleolus and sometimes contain a few peroxidase-positive granules. The N:C ratio in promonocytes is 4:1 to 2:1 (see Fig. 1-45E).²⁴

TABLE 1-7 Morphological Characterization of the Granulocytic (Eosinophilic) Series

Name	Cell	% in BM	Cell Size, μm	N:C Ratio	Nuclear Shape	Nuclear Position	Nuclear Color/ Chromatin	Nucleoli	Color/Amount of Cytoplasm	Cytoplasmic Granules
Myeloblast		0–2%	15–20	7:1–5:1	Round	Eccentric or central	Light, reddish-blue, fine meshwork with no aggregation of material	1–3	Basophilic/scanty	Absent
Promyelocyte		1–4%	12–24	5:1–3:1	Round	Eccentric or central	Light reddish-blue, fine meshwork, slight aggregation may be seen at nuclear membrane	1–2	Basophilic and increased	Present, fine azurophilic, nonspecific granules
Eosinophilic myelocyte		0–3%	10–18	2:1–1:1	Oval or round: slightly indented	Usually eccentric	Reddish-blue fine chromatin with slightly aggregated or granular pattern	May or may not have nucleolus	Bluish-pink/moderate	Present, reddish-orange, uniform (specific) eosinophilic granules
Eosinophilic metamyelocyte		0–5%	10–18	1:1	Usually indented (kidney-shaped)	Central or eccentric	Light blue-purple with basophilic chromatin easily distinguishable	None	Pink/moderate	Present, reddish-orange, uniform (specific) eosinophilic granules
Eosinophilic band		0–5%	10–16	1:1–1:2	Elongated, narrow band shape of uniform thickness	Central or eccentric	Deep blue-purple, coarsely granular chromatin	None	Pink/moderate	Present, red, uniform, (specific) eosinophilic granules
Eosinophilic segmented (eosinophil)		0–1%	10–16	1:3	2 distinct nuclear lobes	Central or eccentric	Deep blue-purple, coarsely granular chromatin	None	Pink/moderate	Present, red, uniform, (specific) eosinophilic granules

% in BM = Percentage of total nucleated cells in bone marrow represent normal reference ranges for adults taken from the University of Texas Health Science Center and University Hospital, San Antonio, Texas.

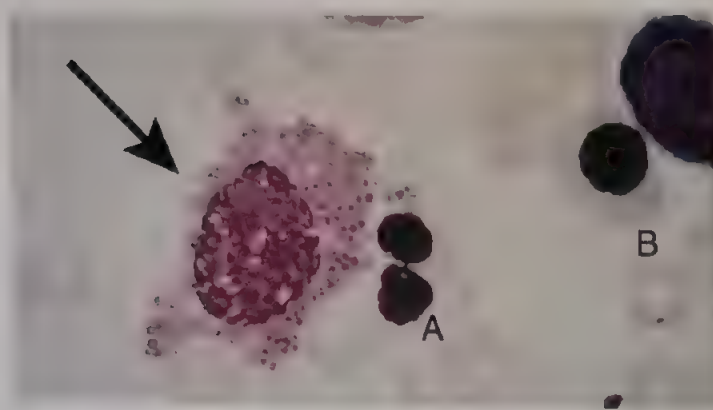


FIGURE 1-43 Arrow: Tissue eosinophil; A. Binucleated orthochromatic normoblast. B. Orthochromatic normoblast.

Promonocytes and monoblasts are not easily identifiable in bone marrow or peripheral blood smears except in disorders in which there is marked proliferation of monocytic cells. The identification of early monocytic cells is based on slightly indented, or folded, large nuclei and on association with more mature cells that have pseudopods and brainlike convolutions in the nucleus.

Monocytes and Macrophages

Promonocytes develop into monocytes. Monocytes enter the circulation for a short time and then migrate into tissue to transform into tissue macrophages.²⁴

The characteristic features of monocytes in normal peripheral blood are given in the earlier part of this chapter (see Fig. 1-10). As monocytes mature, they become too large to pass readily through capillaries, and so they move into tissue and convert into macrophages in many organs (e.g., pulmonary alveolar macrophages, peritoneal macrophages, splenic macrophages, Kupffer cells in the liver, and connective tissue macrophages).²⁰ This transformation involves rapid growth, enlargement, and intensified phagocytic activity. Macrophages do not normally reenter the bloodstream but may reenter the circulation during inflammation.²⁸

Macrophages are large, irregularly shaped tissue cells (25 to 80 μm) with a round or reniform nucleus that contain one or two nucleoli, clumped chromatin, abundant cytoplasm with vacuoles, and numerous azurophilic granules.³ Macrophages are also called histiocytes (histio = tissue; cyte = cell). See Table 1-9 for the morphological characteristics of the monocytic macrophage series.

Lymphopoiesis

The lymphoid progenitor cell is derived from the hematopoietic stem cell. The common lymphoid progenitor cell can differentiate into either T cells, B cells, and natural killer (NK) cells depending on the microenvironment. T cells differentiate in the thymus, B cells in adult bone marrow, and NK lymphocytes are currently considered to originate and mature in the BM.²⁹ Natural killer (NK) cells have a major role in host defense and immune-surveillance.²⁹ However, whether NK cell maturation occurs primarily in the BM niche is still being debated.²⁹ It is speculated that the early phases of NK lymphocyte development occur in the bone marrow, and the later

stages of differentiation can take place in the secondary lymphoid tissues, such as the tonsils, spleen, and lymph nodes.²⁹ It is known that NK lymphopoiesis is supported through interactions with stromal cells, cytokines, growth factors, and other soluble molecules.²⁹ T, B, and NK cells can be distinguished functionally and by immunological marker studies.³⁰ (See the section on CD Nomenclature later in this chapter.)

In primary lymphoid organs such as the thymus and bone marrow, lymphocytes differentiate, proliferate, and mature into fully functional immune cells. In secondary lymphoid organs such as lymph nodes, spleen, and mucosal tissues (tonsils, Peyer's patches), lymphocytes communicate and interact with **antigen-presenting cells (APCs)**, phagocytes, and macrophages in an active immune response.³¹

Lymphoblasts and Prolymphocytes

The earliest lymphocytes are identified as lymphoblasts and prolymphocytes. Lymphoblasts contain a large, round nucleus with a small or moderate amount of basophilic cytoplasm. The nuclear chromatin strands in lymphoblasts are thin, loose, evenly stained, and not clumped. One or several nucleoli are usually demonstrable.²⁴ These cells measure 10 to 20 μm in diameter and have a N:C ratio of 7:1 to 4:1²⁴ (see Fig. 1-45A).

Prolymphocytes have an intermediate chromatin pattern that has clumps in some areas of the nucleus but does not appear as clumped as in mature lymphocytes. **Parachromatin**, which appears reddish-purple, may be present in the nucleus. Nucleoli are less distinct than in lymphoblasts. Prolymphocytes are slightly smaller than lymphoblasts, approximately 9 to 18 μm , and have a N:C ratio of 5:1 to 3:1²⁴ (see Fig. 1-45D). Differences are subtle, and when in doubt, the cell should be called a lymphocyte. The morphological characteristics of the lymphocytic series are summarized and illustrated in Table 1-10.

Lymphocytes

The morphological description of mature lymphocytes may be found in the first part of this chapter.



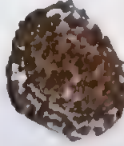
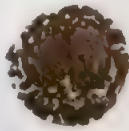
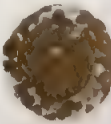

Plasmablasts and Proplasmacytes

Cells designated as **plasmablasts** are similar to blast cells of other series. The nuclei are large in relation to the cytoplasm (N:C ratio is 5:1 to 4:1; see Table 1-12); appear round with fine, linear chromatin strands; and have a clearly visible nucleolus²⁴ (see Fig. 1-45C). The cytoplasm is blue. Plasmablasts are identified primarily in the presence of proplasmacytes and plasmacytes but cannot be easily differentiated from other blasts. The plasmablast appears slightly larger than the more mature plasmacyte. The plasmablast is 16 to 25 μm , and the mature plasma cell is 10 to 20 μm .¹⁴

Proplasmacytes and plasmacytes differ from plasmablasts in that the color of the cytoplasm is deep blue, the juxtanuclear light areas are prominent, and the nuclei are eccentric.³ The chromatin structure of the nuclei in proplasmacytes is intermediate between that of plasmablasts and plasmacytes. In proplasmacytes, the nucleolus may be ill-defined or absent.³ The N:C ratio in proplasmacytes is 4:1 to 3:1 (see Fig. 1-45F).²⁴

Plasmablasts and proplasmacytes, although not observed in normal bone marrow, are seen in diseases associated with

TABLE 1-8 Morphological Characteristics of the Granulocytic (Basophilic) Series

Name	Cell	% in BM	Cell Size, μm	N:C Ratio	Nuclear Shape	Nuclear Position	Nuclear Color/ Chromatin	Nucleoli	Color/Amount of Cytoplasm	Cytoplasmic Granules
Myeloblast		0–2%	15–20	7:1–5:1	Round	Eccentric or neutral	Light, reddish-blue, fine meshwork with no aggregation of material	1–3	Basophilic/slight	Absent
Promyelocyte		1–4%	12–24	5:1–3:1	Round	Eccentric or central	Light reddish-blue meshwork, slight aggregation at nuclear rim	1–2	Basophilic/increased	Present, fine azurophilic, non-specific granules
Basophilic myelocyte		0–1%	10–18	2:1–1:1	Oval or round; slightly indented	Commonly eccentric, may be central	Reddish-blue, fine chromatin with slightly aggregated or granular pattern	May/may not have nucleolus	Bluish pale, moderate	Present, coarse (specific) basophilic, nonuniform granules
Basophilic metamyelocyte		0–1%	10–18	1:1	Usually indented (kidney-shaped), oval	Central or eccentric	Light blue-purple with basophilic chromatin easily distinguishable	None	Pale blue, moderate	Present, coarse violet-blue, nonuniform granules
Basophilic band		0–1%	10–16	1:1–1:2	"Sausage-shaped"	Central or eccentric	Deep blue-purple, coarsely granular chromatin	None	Pale blue, moderate	Present, coarse, violet-blue, nonuniform granules
Basophilic segmented (basophil)		0–1%	10–16	1:3	Two distinct nuclear lobes	Central or eccentric	Deep blue-purple, coarsely granular chromatin	None	Pale blue, moderate	Present, coarse, violet-blue, nonuniform granules

% in BM = Percentage of total nucleated cells in bone marrow represent normal reference ranges for adults taken from the University of Texas Health Science Center and University Hospital, San Antonio, Texas.

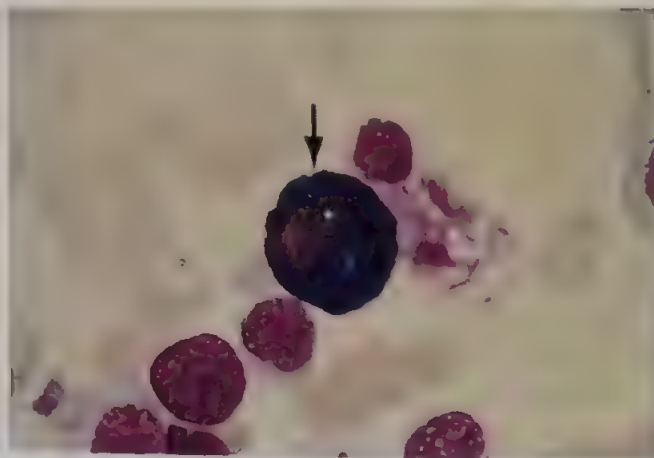


FIGURE 1-44 Tissue basophil (arrow).

abnormal immunoglobulin production, especially multiple myeloma.²⁴

Plasmacytes (Plasma Cells)

Plasmacytes represent the end stage of antigen-activated B-lymphocytes in secondary lymphoid organs.³² They are not observed in the peripheral blood smears of normal individuals but constitute about 1% of the nucleated cells in normal marrow.²⁴ Mature plasmacytes range in size from 10 to 20 μm .²⁴ They may be round or oval, with slightly irregular margins.

The cytoplasm is nongranular and usually stains a deep or vibrant blue. The cytoplasm adjacent to the nucleus is pale, with a perinuclear clear zone containing the Golgi apparatus, and at the cell periphery there are secretory vesicles.³³ Fibrillar structures that stain blue may be demonstrable in the cytoplasm. One or several small vacuoles may be

observed. There is no evidence of phagocytosis of visible particles.³

The nucleus of a plasmacyte is relatively small, round, or oval, and eccentrically placed in the cell. The nuclear chromatin is clumped or coarse and lumpy, similar to that of a lymphocyte (see Figs. 1-45I, 1-46, and 1-47). The N:C ratio of a plasma cell is 1:1 to 1:2.³⁴

Immune globulins manufactured by plasmacytes produce unusual morphological variants. The proteinaceous material that appears in the form of round globules are often red or pink, but may be blue or almost colorless, and are called Russell bodies.³⁴ The morphological characteristics of the plasmacytic series are summarized and illustrated in Table 1-11.

Megakaryocytopoiesis

The megakaryocyte is the largest hematopoietic cell in the bone marrow and descends from the same multipotent stem cell as do the other blood cells. The mission of megakaryocytes is to proliferate and then fragment their cytoplasm into platelets, when needed, to maintain a normal number of platelets (150,000 to 400,000/ μL). The maturation of the megakaryocyte involves endomitosis, which is a process whereby the chromosome material reduplicates but the nucleus does not divide.³⁵ The result is a polyploid nucleus. Each nuclear reduplication causes a doubling of the nuclear material. The cytoplasm increases in amount and number of granules, but it does not divide. Megakaryoblasts are moderately sized cells in the range of 20 to 45 μm with a single, round (or slightly oval), primitive nucleus; one or two nucleoli; and blunt protrusions that stain blue and may contain chromophobic globules.²⁸ The scanty cytoplasm is nongranular and basophilic. The N:C ratio is 5:1 to 3:1 (Table 1-12).¹⁴ The megakaryocytic series is shown in Figure 1-48.

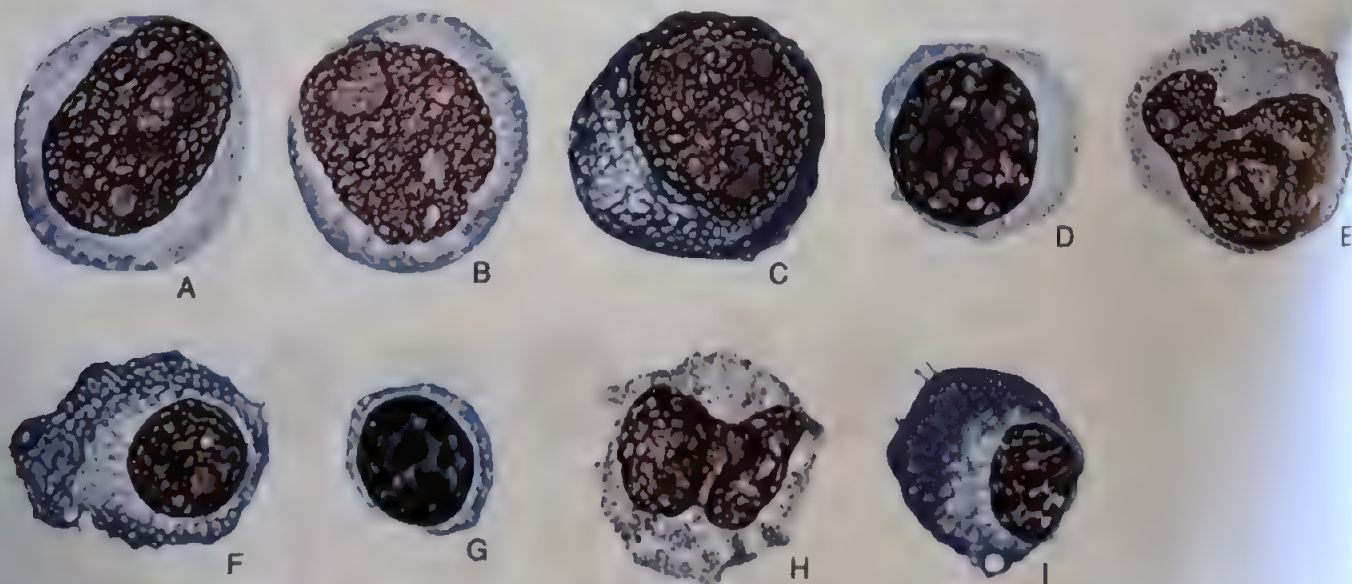


FIGURE 1-45 Lymphocytic, monocytic, and plasmacytic systems. A. Lymphoblast. B. Monoblast. C. Plasmablast. D. Prolymphocyte. E. Promonocyte. F. Proplasmacyte. G. Lymphocyte with clumped chromatin. H. Monocyte. I. Plasmacyte. (From Diggs LW, et al. *The Morphology of Human Blood Cells* ed 5. Abbott Park, IL: Abbott Laboratories; 1985, pp 1-18, 25-27, with permission.)

TABLE 1-9 Morphological Characteristics of the Monocytic Series




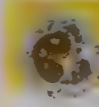



Name	Cell	% in BM	Cell size, μm	N:C Ratio	Nuclear Shape	Nuclear Position	Nuclear Color/ Chromatin	Nucleoli	Color/Amount of Cytoplasm	Cytoplasmic Granules
Monoblast		0–1%	15–25	7:1–4:1	Round, oval, or slightly folded	Eccentric	Pale red-purple, fine, thready chromatin	1–2	Basophilic/moderate	None
Promonocyte		0–1%	14–20	4:1–2:1	Round with chromatin creases or cerebriform folding, more distinct	Central	Pale red-purple, reticular pattern	0–2	Paler gray basophilic/abundant with “bleb-like” pseudopodia at border	May/may not contain fine, red, ductlike particles
Mature monocyte		0–2%	12–18	1:1–2:1	Increased folding or elongated	Central	Blue-purple, finer reticular pattern than immature forms	None	Pale gray-blue/abundant “bleblike” pseudopodia	Numerous fine, pale red, dustlike particles throughout cytoplasm
Macrophage			25–80	1:2 or 1:3	Round or reniform	Eccentric	Clumped chromatin	1–2	Abundant with vacuoles	Numerous azurophilic granules

TABLE 1-10 Morphological Characteristics of the Lymphocytic Series

Name	Cell	Cell size, μm	N:C Ratio	Nuclear Shape	Nuclear Position	Nuclear color/Chromatin	Nucleoli	Color/Amount of cytoplasm	Cytoplasmic Granules
Lymphoblast		10–20	7:1–4:1	Round	Eccentric or central	Undifferentiated red-purple/smooth chromatin	1–2	Clear basophilic/scanty	Absent
Prolymphocyte		9–18	5:1–3:1	Round or indented	Eccentric with scanty cytoplasm to one side or round	Condensed, clumped blue-purple chromatin with red-purple parachromatin	0–1	Clear basophilic/scanty	Absent
Mature lymphocyte		7–15	4:1–2:1	Round or indented	Eccentric with scanty cytoplasm to one side or round	Homogenous, coarse blue-purple nuclear chromatin	None	Light sky blue/scanty to moderate	Usually absent, few azurophilic granules seen occasionally

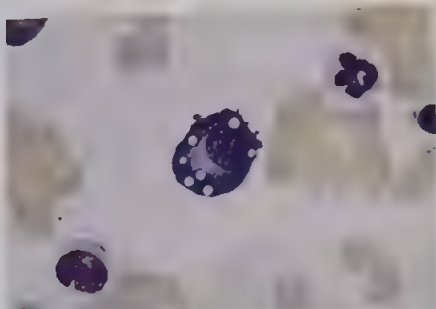


FIGURE 1-46 Center: Plasma cell; upper right: Segmented neutrophil; lower left: Resting monocyte.

Pathological alterations in megakaryoblasts are observed in several neoplastic diseases. The presence of micromegakaryoblasts is typical of acute megakaryocytic leukemia (see Chap. 17). Micromegakaryoblasts are small and difficult to distinguish from myeloblasts, but cytoplasmic blebs or budding (suggesting early platelet formation) helps to identify micromegakaryoblasts.³⁶

As the megakaryoblast matures into a promegakaryocyte, it increases both the amount of nuclear material and the amount of cytoplasm itself (N:C ratio is 3:1 to 1:1).³⁵

A promegakaryocyte not only increases the size of the nucleus but also becomes lobulated, with each lobe

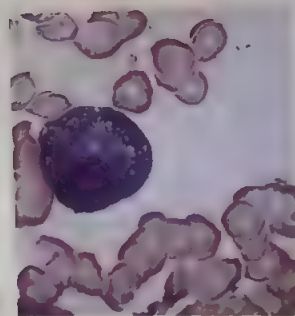





FIGURE 1-47 Plasma cell.

having a $2n$ complement of DNA. The size of the promegakaryocyte ranges from 20 to 80 μm .³⁶ Reddish granules appear in the enlarging bluish cytoplasm. Electron micrographs reveal that demarcation membranes are beginning to develop as invaginations from the plasma membrane of the megakaryocyte. The demarcation membrane system establishes an outer limit of each platelet, which is released as a cytoplasmic fragment³⁵ (see Fig. 1-48). As endomitosis and DNA synthesis cease and maximum nuclear number (ploidy) is attained, the megakaryocyte has increased in volume with an abundant amount of pinkish cytoplasm and a multilobulated nucleus (Figs. 1-49 through 1-53). The

TABLE 1-11 Morphological Characteristics

Name	Cell	Cell Size, μm
Plasmablast		16–25
Proplasmacyte		15–20
Mature plasma cell		10–20


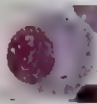
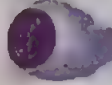
nuclei of megakaryocytes are connected by strands. They have irregular nuclear shapes, and the size of the megakaryocyte ranges from 20 to 80 μm . The majority of the cells are of the $8n$ class (16n average ploidy represents tetraploid). Nuclear chromatin is linear and coarse. Nucleoli are not distributed, dense granules that stain blue. The demarcation membrane system is not yet formed. The lumen open, the cytoplasm is divided into segments that define platelet limits. The N:C ratio of development ranges from 3:1 to 1:1. The morphological characteristics of the megakaryocyte are shown in Table 1-12.

After maturation is completed, the megakaryocyte undergoes membrane ruptures, the entire megakaryocyte fragments, and thrombopoiesis occurs. The naked nucleus (Fig. 1-54) is soon phagocytosed by a macrophage.

Some mature megakaryocytes extend into the marrow sinuses and extend portions of their cytoplasm through the basement membrane into the sinusoidal cells of the marrow sinusoids. Membrane bound platelets are released into the bloodstream from these cells. Further fragmentation to form individual platelets occurs after release into the sinus. One megakaryocyte can release several thousand platelets.

In marrow smears of normal individuals, approximately 1 to 4 megakaryocytes per field are seen. These cells are in the late stage of maturation.

TABLE 1-11 Morphological Characteristics of the Plasmacytic Series

Name	Cell	Cell Size, μm	N:C Ratio	Nuclear Shape	Nuclear Position	Nuclear color/ Chromatin	Nucleoli	Color/ Amount of cytoplasm	Cytoplasmic Granules
Plasmablast		16–25	5:1–4:1	Round	Central	Pale red-purple, fine stippled chromatin	1–3	Pale blue/scanty to moderate frequent perinuclear clear zone	None
Proplasmacyte		15–20	4:1–3:1	Round or oval	Eccentric	Red-purple, increased granularity of chromatin	0–1	Dark blue/moderate	None
Mature plasma cell		10–20	1:1–1:2	Round or oval	Usually eccentric	Blue-purple, dense chromatin with large clumps near nuclear margin	None	Dark blue/moderate cytoplasm with perinuclear clear zone, may contain vacuoles	None

nuclei of megakaryocytes are connected by a nuclear strand, have irregular nuclear shapes, and may be superimposed. The size of the megakaryocyte ranges from 30 to 100 μm .³⁶ The majority of the cells are of the $8n$, $16n$, and $32n$ ploidy classes ($16n$ average ploidy represents eight lobes). The chromatin is linear and coarse. Numerous small, uniformly distributed, dense granules that stain reddish-blue are present. The demarcation membrane system is uniform and its lumen open; the cytoplasm is divided into partitions that define platelet limits. The N:C ratio at the megakaryocyte stage of development ranges from 1:1 to 1:2.³⁵ The morphological characteristics of the megakaryocytic series are shown in Table 1-12.

After maturation is completed, the megakaryocyte membrane ruptures, the entire megakaryocyte cytoplasm fragments, and thrombopoiesis occurs.³⁵ The polyploid naked nucleus (Fig. 1-54) is soon to be engulfed by a macrophage.

Some mature megakaryocytes are located adjacent to marrow sinuses and extend portions of their cytoplasm through the basement membrane and between endothelial cells of the marrow sinusoids to put platelets into the sinus. Membrane-bound platelets are released and swept into the bloodstream from these cytoplasmic projections. Further fragmentation to form individual platelets occurs after release into the sinus. One megakaryocyte can release several thousand platelets.³⁷

In marrow smears of normal individuals, there are approximately 1 to 4 megakaryocytes per 100 nucleated cells, and these cells are in the late stage of maturation.³⁵ See Table 1-12

for the morphological characteristics of the megakaryocytic series.

Bone-Derived Cells

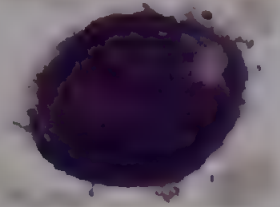
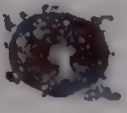
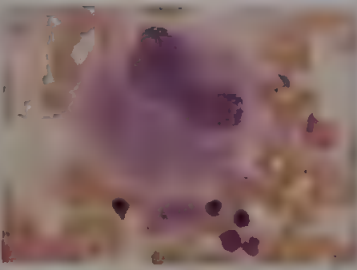

The formation of the bone marrow cavity results from a complex process in which hematopoietic cells migrate and colonize spaces originally occupied by cartilage and bone. This process occurs both in the long bones of the limbs and the membranous bones of the skull, which develop directly into bone.²⁴ There are three major cell types in bone; osteoblasts, osteoclasts, and osteocytes. These cells form and maintain bone and secrete metabolic factors. Studies have shown that bone formation and hematopoiesis are closely linked, in that the degree of hematopoiesis correlates with the rate of bone turnover.^{38,39} The osteoblast and the osteoclast are cells that exhibit different functions yet play essential roles in the formation of the bone cavity.³⁹

Osteoblasts

An **osteoblast** is a large cell that can measure up to 30 μm , with ample cytoplasm and a small, round, eccentrically placed nucleus.¹⁴ These cells may be traumatized in the process of marrow aspiration and smearing, and often have irregular shapes and cytoplasmic streamers. The nuclear chromatin strands and nuclear margins are well defined and stain purple-red (Fig. 1-55).

Throughout the blue cytoplasm, there are small spherical bodies that are colorless and give a bubbly appearance to the cytoplasm. Within the cytoplasm, there is a prominent round or oval chromophobic zone that stains lighter than the rest of

TABLE 1-12 Morphological Characteristics of the Megakaryocytic Series

Name	Cell	Cell size, μm	N:C Ratio	Nuclear Shape	Nuclear Position	Nuclear color/ Chromatin	Nucleoli	Color/Amount of Cytoplasm	Cytoplasmic Granules
Megakaryoblast		20–45	5:1–3:1	Usually single round, oval, indented or kidney-shaped	Central or eccentric	Red-purple chromatin with distinct chromatin	1–2	Basophilic, pseudopodia frequent/scanty	Nongranular
Late Promegakaryocyte		20–80	3:1–1:1	Usually single round, oval indented or kidney-shaped	Central or eccentric	Red-purple increased granularity of nuclear chromatin	0–1, usually less than megakaryoblast	Basophilic/abundant with pseudopodia	Fine azurophilic granules
Megakaryocyte		30–100	1:1–1:2	Lobulated (2 or more lobes)	Central	Blue-purple, granular	None	Pale blue with pink cast/abundant	Numerous fine azurophilic granules
Thrombocyte (platelets)		1–4	n/a	n/a	n/a	n/a	n/a	Light blue, fragment of megakaryocyte cytoplasm	Reddish-blue, fine, evenly dispersed

n/a = Not applicable.

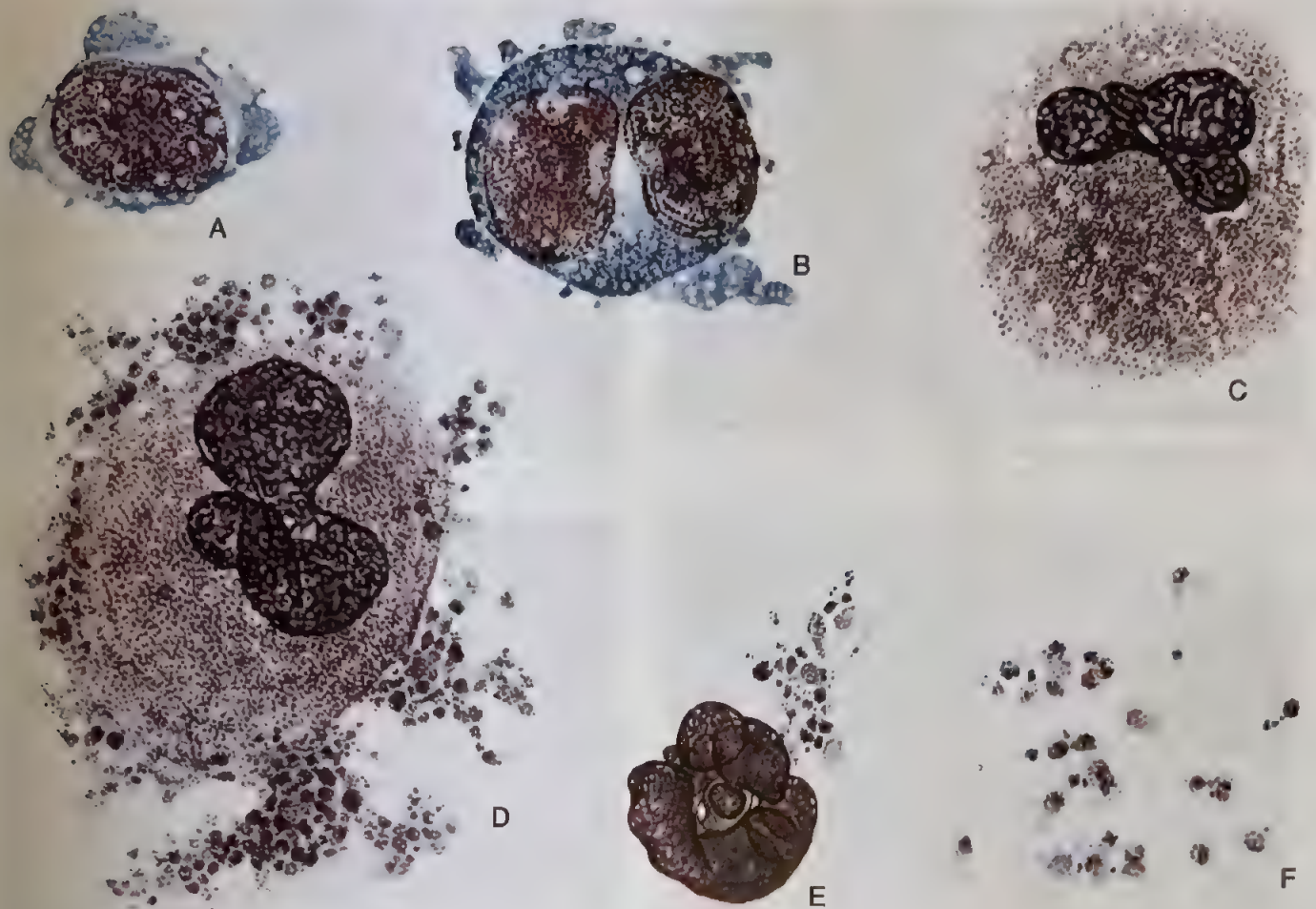


FIGURE 1-48 Megakaryocytic system. **A.** Megakaryoblast with single oval nucleus, nucleoli, and bluish foamy marginal cytoplasmic structures. **B.** Promegakaryocyte with two nuclei, granular blue cytoplasm, and marginal bubbly cytoplasmic structures. **C.** Megakaryocyte with granular cytoplasm and without discrete thrombocytes (platelets). **D.** Megakaryocyte with multiple nuclei and with thrombocytes (platelets). **E.** Megakaryocyte nucleus with attached thrombocytes. **F.** Thrombocytes (platelets). (From Diggs, LW, et al: *The Morphology of Human Blood Cells*, ed. 5, 1985, pp 33-34, 48-50, 85. Abbott Laboratories, Abbott Park, IL, with permission.)

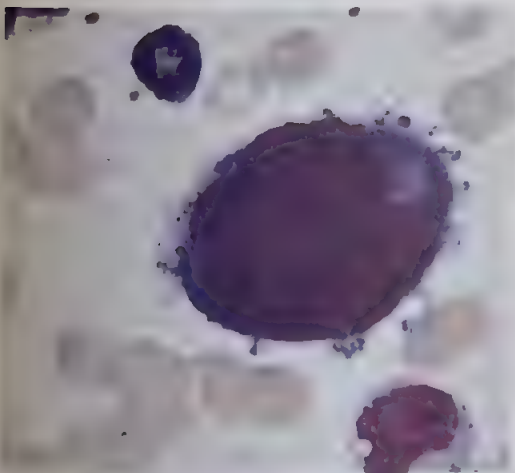


FIGURE 1-49 Center: Early megakaryocyte.

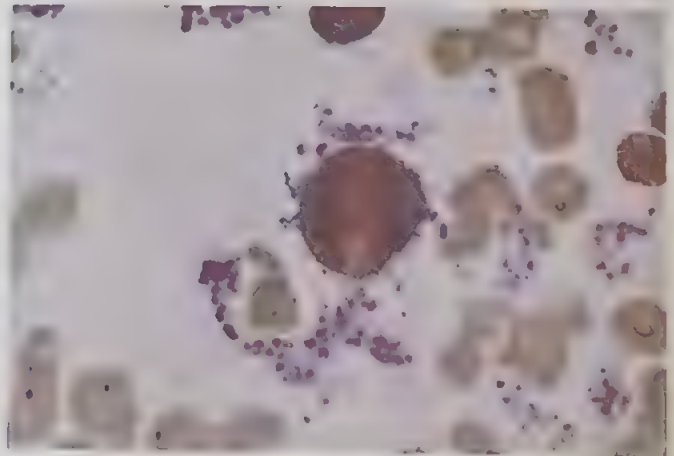


FIGURE 1-50 Center: Early megakaryocyte.

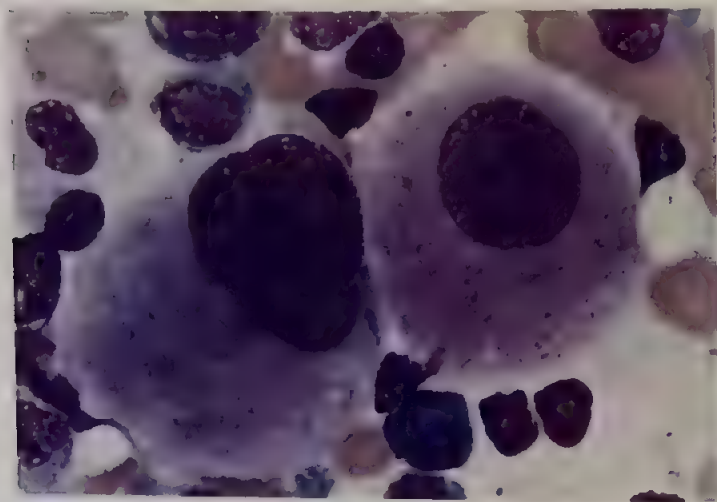


FIGURE 1-51 Megakaryocytes without platelets.

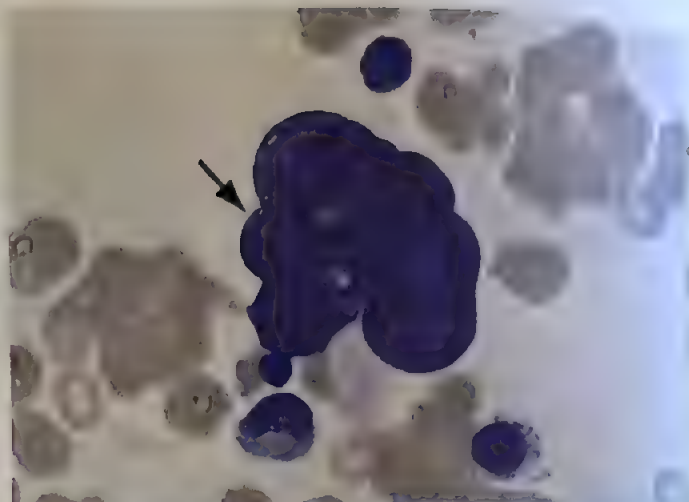


FIGURE 1-54 Naked nuclei, megakaryocyte.

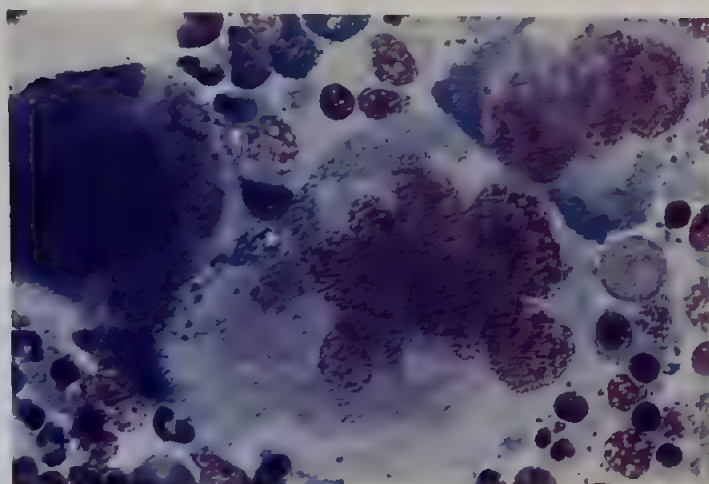


FIGURE 1-52 Megakaryocytes tend to be in small groups with multi-lobulated single nuclei. Mature megakaryocytes have numerous fine cytoplasmic granules, and occasionally platelet units can be seen at their periphery (magnification $\times 640$).

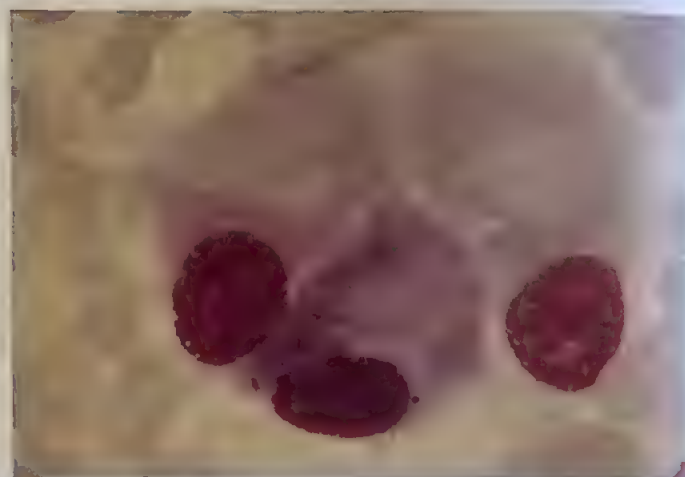


FIGURE 1-55 Three osteoblasts.

the cytoplasm. This area is usually away from the nucleus but may be adjacent to it.

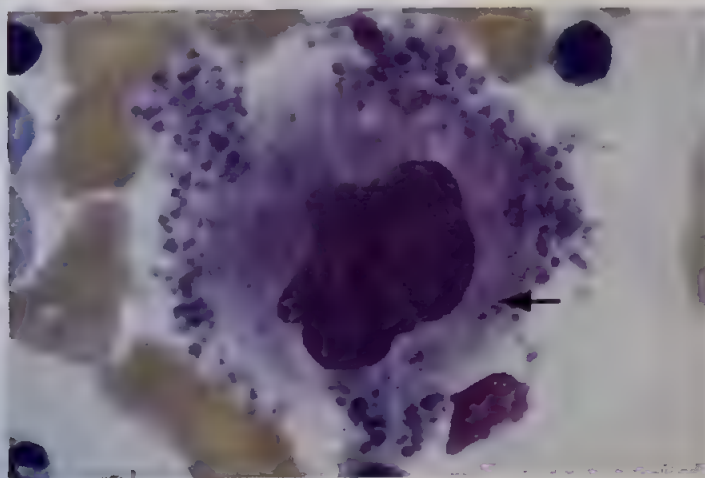


FIGURE 1-53 Megakaryocyte with platelets.

ADVANCED CONTENT

Osteoblasts, more often seen in marrow from young children, are responsible for the formation, calcification, and maintenance of trabeculae and cancellous bone. Osteoblasts originate from mesenchymal progenitor cells and produce bone matrix proteins, which contribute to the structure of the bone and stromal matrix.⁴⁰ A matrix protein that regulates bone mineralization, now referred to as an osteoblast-derived factor, is osteocalcin.⁴¹ Osteocalcin has been suggested to be an osteoblast-derived endocrine hormone that regulates multiple target organs.⁴¹ Bone is now considered an endocrine organ that secretes a growing number of hormones.⁴²

Osteoblasts have irregular shapes, eccentric nuclei, cytoplasmic protrusions, blue cytoplasmic fibrils, and vacuoles. The chromophobic zone of the osteoblast is often separate from the nuclear margin and, when adjacent to the nucleus, does not surround or enclose the nucleus.

Osteoblasts that occur in clusters or aggregates may be misinterpreted as malignant cells (Fig. 1-56). Malignant

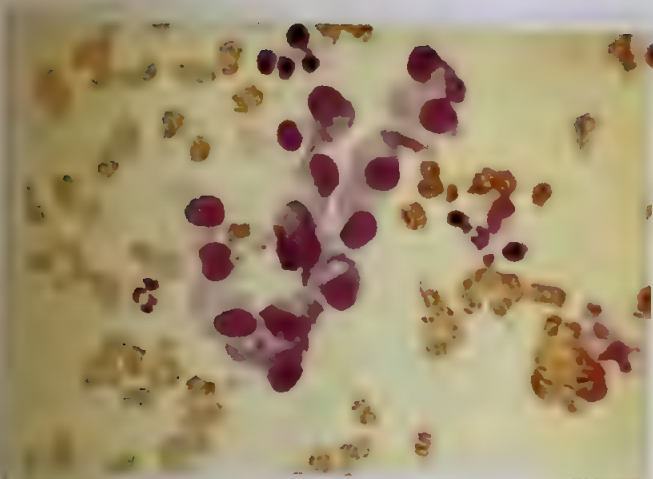


FIGURE 1-56 Group of osteoblasts (center) aspirated from the marrow of a child (magnification $\times 400$).

cells in a cluster are crowded and distorted, with indistinct margins, rendering it impossible to identify individual cells. Individual osteoblasts in a cluster can usually be identified. The size, shape, structure, and color of malignant cells are variable, whereas osteoblasts are more orderly and uniform. Chromophobic areas in the cytoplasm of osteoblasts are seldom demonstrable in malignant cells.

The osteocyte is a bone matrix-embedded cell that regulates bone homeostasis. Two very powerful proteins are secreted by the osteocyte: sclerostin, which inhibits bone formation, and a receptor-activator of a cytokine (RANKL), which is required for the formation of osteoclasts.⁴³

Recent studies report another function of the osteocytes, in which they secrete factors that control hematopoiesis and the hematopoietic stem and progenitor cells (HSPC) niche. However, the exact mechanism is not completely understood.⁴³

Osteoclasts

Osteoclasts are giant (greater than 100 μm), multinucleated, irregularly shaped marrow phagocytes that are capable of reabsorption of bone and are derived from the monocyte-macrophage hematopoietic cell lineage.⁴⁴ Osteoclasts have from 2 to 50 nuclei, which are separate, usually round or oval, uniform in size, and haphazardly distributed within the cytoplasm⁴⁵ (Fig. 1-57). The abundant cytoplasm with ragged margins is bluish, with numerous reddish lysosomal granules containing acid phosphatase.⁴⁵

Osteoclasts are large with granular cytoplasm, irregular shapes, and multiple nuclei. The nuclei of osteoclasts are separated, uniform in size, and have no visible connections to each other (see Figs. 1-57 and 1-58).⁴⁵

Osteoclasts secrete enzymes that aid in dissolution of osteoid tissue and calcified bone.⁴⁵ These cells are involved in the degradation (or reabsorption) of bone, which is essential for the formation of the bone marrow cavity and bone remodeling. Osteoclasts adhere to the bone matrix and secrete lytic enzymes that degrade it. Osteoclast proliferation and survival of their precursors depend on the cytokine, macrophage colony-stimulating factor (M-CSF).⁴⁴ The morphological

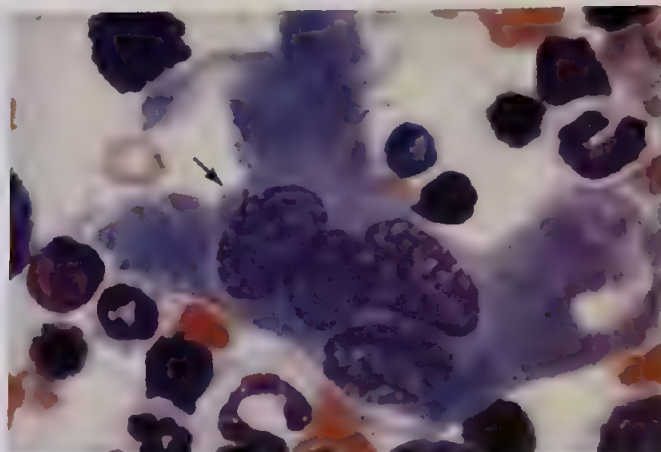


FIGURE 1-57 The osteoclast is usually seen as a single giant cell with multiple and separated nuclei and basophilic granular cytoplasm (center) (magnification $\times 640$).

MEGAKARYOCYTE

OSTEOCLAST

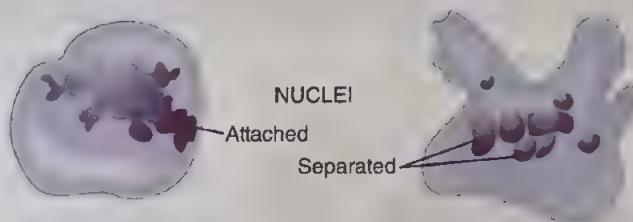


FIGURE 1-58 Osteoclast versus a megakaryocyte.

characteristics of the osteoblasts and osteoclasts are illustrated and compared in Table 1-13.

The morphological characteristics of the multinucleated osteoclasts and the multilobulated megakaryocytes are sometimes difficult to distinguish and are illustrated and compared in Table 1-14.

Cell Line Ontogeny (Evolution)

Multipotent Stem Cells—Colony-Forming Units (CFUs) (Hematopoietic Stem Cell)

As described earlier, all blood cells come from an unrecognized pluripotent stem cell. The pluripotent stem cell has the capacity for continuous self-replication and differentiation into a **multipotent progenitor stem cell (MPP)**.⁴⁶ The MPP becomes committed to support progenitor cells for myelopoiesis, erythropoiesis, monopoiesis, megakaryopoiesis, and lymphopoiesis.⁴⁶

The multipotential stem cell was shown to exist in a classic experiment in 1961 by Till and McCulloch, who irradiated mice to empty the hematopoietic organs and then injected a suspension of marrow cells intravenously. About a week later, nodules of injected marrow could be observed on the cut surface of the spleen colonies. All cell lines found in normal marrow were generated from the multipotential stem cells in the marrow suspension. The multipotential stem cell giving rise to several cell lines was called the colony-forming unit—granulocyte-erythrocyte-macrophage-megakaryocyte (CFU-GEMM).⁴⁷ The CFU-GEMM in a

TABLE 1-13 Morphological Characteristics of Osteoblasts and Osteoclasts



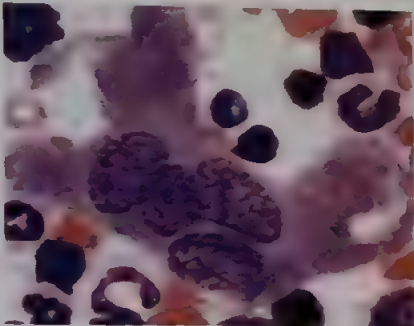
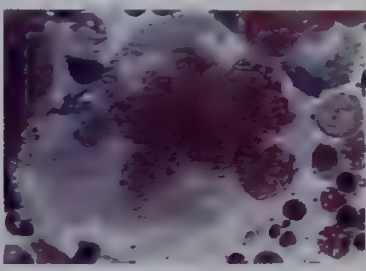
Name	Cell	Size, μm	Nuclei	Nucleolus	Cytoplasm	Shape
Osteoblast		Up to 30	Round, eccentric, uninuclear	Present	Chromophobic area, usually away from nucleus	Resemble plasmacytes
Osteoclast		>100	Multinucleated, uniform size	Present	Bluish, reddish lysosomal granules, cytoplasmic protrusions	May be confused with megakaryocytes

TABLE 1-14 Morphological Characteristics of Osteoclasts and Megakaryocytes

Name	Cell	Cell Size, μm	Shape	Cytoplasm	Nuclei
		>100	Irregular	Granular	Multiple, uniform in size, unconnected by nuclear strands
Osteoclast					
		30–100	Irregular	Granular	Multilobed, connected by nuclear strands, not uniform in size
Megakaryocyte					

colony assay forms a series of progenitor cells (CFU-GM, CFU-Eo, CFU-Bas, CFU-Meg, BFU-E, CFU-E) under appropriate growth conditions. Box 1-2 lists relevant abbreviations and acronyms. CFU-GM makes colonies of granulocytes and monocytes and/or macrophages (Fig. 1-59). CFU-Eo forms colonies of eosinophils. CFU-Bas makes early basophils and mast cells. CFU-Meg forms megakaryocyte colonies. There are two colonies of erythroid progenitor cells: the early burst-forming unit-erythroid (BFU-E) (Fig. 1-60) and the more mature colony-forming unit-erythroid (CFU-E).⁴⁷

The common lymphoid progenitor stem cell is also derived from the MPP. The common lymphoid progenitor stem cell has the potential to differentiate into progenitors for T, B, or NK lymphocytes.⁴⁹ T cells participate in immune functions of a cellular nature, either directly cytotoxic, or by helping or suppressing immune activities through interaction with other immunocompetent cells. B cells differentiate into plasmacytes, which secrete specific immunoglobulins important in the host's defense against infection. Another population of lymphocytes, called natural killer (NK) cells, have none of the characteristics of either the T or the B cells. NK cells

BOX 1-2 Relevant Abbreviations and Acronyms

APC	antigen-presenting cells
BFU-E	burst forming unit-erythrocyte
BM	bone marrow
CD	clusters of differentiation
CFU	colony-forming units
CFU-Bas	colony-forming unit-basophil
CFU-E	colony-forming unit-erythrocyte
CFU-Eo	colony-forming unit-eosinophil
CFU-GEMM	colony-forming unit-granulocyte, erythrocyte, monocyte-macrophage, megakaryocyte
CFU-GM	colony-forming unit-granulocyte, monocyte-macrophage
CFU-Meg	colony-forming unit-megakaryocyte
CMP	common myeloid progenitor
CSF	colony-stimulating factor
HPC	hematopoietic progenitor cell
HSC	hematopoietic stem cell
HSPC	hematopoietic stem and progenitor cells
IL	interleukin
LT-HSC	long-term HSC
ST-HSC	short-term HSC
mAb	monoclonal antibody
M-CSF	macrophage colony-stimulating factor
MBP	major basic protein
MEP	megakaryocyte-erythroid progenitor
MSC	multipotent stem cell
MPP	multipotent progenitor stem cell
MPS	mononuclear phagocyte system
N:C ratio	nuclear-to-cytoplasm ratio
NK	natural killer
rHuEPO	recombinant human erythropoietin
rHuG-CSF	recombinant human granulocyte CSF
SCF	stem cell factor
TF	transcription factor
TGF	transforming growth factor
TPO	thrombopoietin

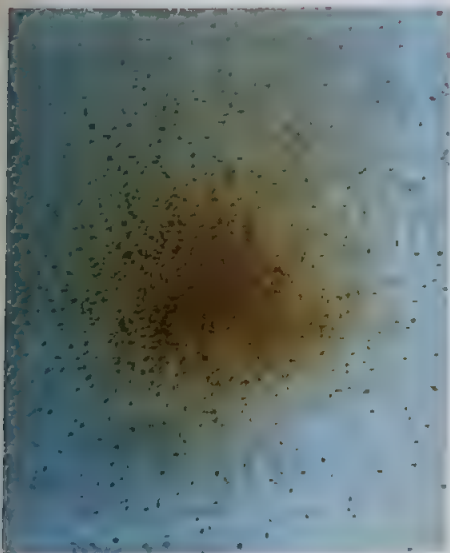


FIGURE 1-59 CFU-GM at 14 days ($\times 50$ magnification). Colony-forming unit that makes colonies of granulocytes, monocytes, and/or macrophages under appropriate growth conditions.

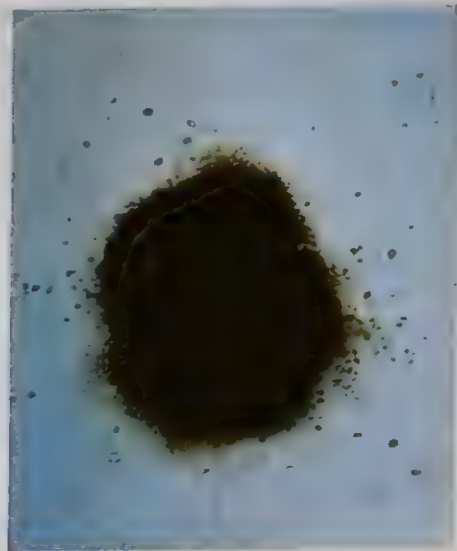


FIGURE 1-60 BFU-E at 18 days ($\times 75$ magnification). Early burst-forming unit, an erythroid progenitor committed to making colonies of erythroid cells.

are innate lymphocytes that play a major role in host defense and immune-surveillance.²⁹ The NK lymphocytes may interact with antibodies to cause destruction of antibody-coated targets or lyse target cells through direct cytotoxic activity.²⁹ B-cell lymphopoiesis is completed in the bone marrow, and T-cell lymphopoiesis occurs in the thymus. NK lymphopoiesis is still being debated but is thought to occur primarily in the bone marrow niche.²⁹

The different types of lymphocytes and their functions are listed in Table 1-15.

Colony-Stimulating Factors and Interleukins

Each cell line is dependent on **cytokines**, which are soluble mediators secreted by cells for the purpose of cell-to-cell communication. Cytokines are secreted glycoproteins that act as intercellular messengers, inducing proliferation, differentiation, growth, or **apoptosis (cell death)** of their target cells.¹⁰ More than 50 cytokines orchestrate hematopoiesis, induce inflammation, and control the immune response. As described earlier, prominent cytokines involved in hematopoiesis include interleukins (ILs), colony-stimulating factors (CSFs), interferons, EPO, and thrombopoietin (TPO). The different cell types and the cytokines they produce are listed in Table 1-16. The characteristics of cytokines are listed in Box 1-3.

TABLE 1-15 Functions of Lymphocytes

Lymphocyte	Function
T cell	Cell-mediated immunity
B cell	Humoral immunity
Natural killer (NK) cells	Direct cytotoxic activity and antibody-dependent cell-mediated lysis

TABLE 1-16 Cellular Cytokine Production of Reported Cell Types

Cell Type	Cytokine*
Endothelial cell	GM-CSF, G-CSF, M-CSF, IL-1, IL-6, TNF- α , CXCL12
Mesenchymal stem cell	SCF, CXCL12, G-CSF, GM-CSF
Monocyte-macrophage	GM-CSF, G-CSF, M-CSF, IL-1, IL-3, IL-6, EPO, SCF, M-CSF, TNF- α
T cell	GM-CSF, IL-2, IL-3, IL-4, IL-5, IFN- γ , TNF- α
Fibroblasts	GM-CSF, G-CSF, M-CSF, IL-6, SCF
NK cells	GM-CSF, TNF- α
Osteoblasts	IL-6, GM-CSF, IL-7
PMN	G-CSF, GM-CSF
B cells	GM-CSF, M-CSF, IL-2, IL-5, IL-6
Marrow stroma	GM-CSF, G-CSF, M-CSF, IL-6, IL-3, SCF, IL-11
Renal parenchyma, liver, and marrow cells	EPO, TPO (hepatocyte)

IL = interleukin; GM-CSF = granulocyte macrophage colony-stimulating factor; G-CSF = granulocyte colony-stimulating factor; M-CSF = macrophage colony-stimulating factor; TNF = tumor necrosis factor; CXCL = chemokine ligand; EPO = erythropoietin; SCF = stem cell factor; IFN = interferon; TPO = thrombopoietin.

BOX 1-3 Cytokine Characteristics

- Glycoproteins
- Produced by many cell types
- Usually act on multiple cell lineages
- Interact synergistically with one another
- Activate receptors at very low concentrations
- Usually act on the neoplastic counterpart of normal target cells
- Usually act throughout the maturation hierarchy from stem cell to the terminally differentiated cell

Cytokines act on multipotent stem cells to stimulate their proliferation and differentiation to committed cell lines (Fig. 1-61).⁴⁰ Cytokines are necessary for a cell to develop from a multipotent stem cell to a progenitor stem cell to a myeloblast, monoblast, erythroblast, lymphoblast, or megakaryoblast.⁴⁰ Some of the cytokines involved in hematopoietic blood cell development are outlined in Table 1-17, with their sources, target cells that they stimulate, and major functions.^{50,51,52,53}

CSFs and interleukins regulate blood cell development by mediating proliferation, differentiation, and maturation of hematopoietic progenitor cells. Box 1-4 lists the six groups of contrasting cytokines.⁵

Trends in Therapeutic Manipulation of Hematopoiesis**Recombinant Cytokines**

Many growth factors have been isolated, biochemically characterized, purified, genetically cloned, and produced through recombinant DNA technology. Many growth factors including G-CSF, GM-CSF, Epo, and the interleukins have been used for clinical application.⁵⁴⁻⁵⁷ In vivo, the regulation of hematopoiesis is under the control of cytokine production in the basal state, maintaining normal blood counts, and during the antigen stimulus state, eliciting cytokine stimuli above the basal state to combat infection. CSFs have been used to strengthen patients with cancer and acquired immunodeficiency syndrome (AIDS), and to guard against infection in bone marrow transplantation recipients.^{55,56} These factors have also been used to treat patients with anemia caused by either surgery or kidney failure.⁵⁷ The blood counts of autologous donors can be raised for donation before surgical procedures. Interleukins are used clinically for wound healing, activating lymphocytes, and assisting in the growth of transplanted or damaged bone marrow.⁵⁷

CRITICAL THINKING QUESTION

- 1-3 Why does treatment with colony-stimulating factors (CSFs) work for patients with cancer, immunodeficiency syndromes, or anemia?

ADVANCED CONTENT**Clinical Trials of Recombinant Cytokines**

Clinical trials of recombinant cytokines using biological substances similar to those in the human body have provided new opportunities for evaluating their clinical usefulness in the treatment of hematologic and oncologic disorders.^{58,59} Investigations have shown that recombinant human granulocyte CSF (rHuG-CSF) accelerates recovery from neutropenia induced by myelotoxic chemotherapy for different types of carcinoma.^{60,61} This recombinant CSF has been given to patients receiving myelosuppressive chemotherapy and undergoing autologous bone marrow transplantation to accelerate the rate of neutrophil recovery.⁶⁰ Clinical trials are being conducted to determine whether rHuG-CSF is effective in correcting severe neutropenia in hematopoietic malignancies, such as hairy-cell leukemia, and also in nonneoplastic hematopoietic diseases, such as aplastic anemia and cyclic neutropenia.⁶²

Among patients with chronic anemia, treatment with recombinant human erythropoietin (rHuEPO) has increased RBC production and alleviated anemia in the majority of patients.⁶² The rise in hematocrit is dose-dependent and in proportion to the increase in RBC mass.⁶² Clinical trials using other synthesized cytokines are currently in progress to determine activity in controlling hematopoiesis. Each factor needs to be purified and its function determined.

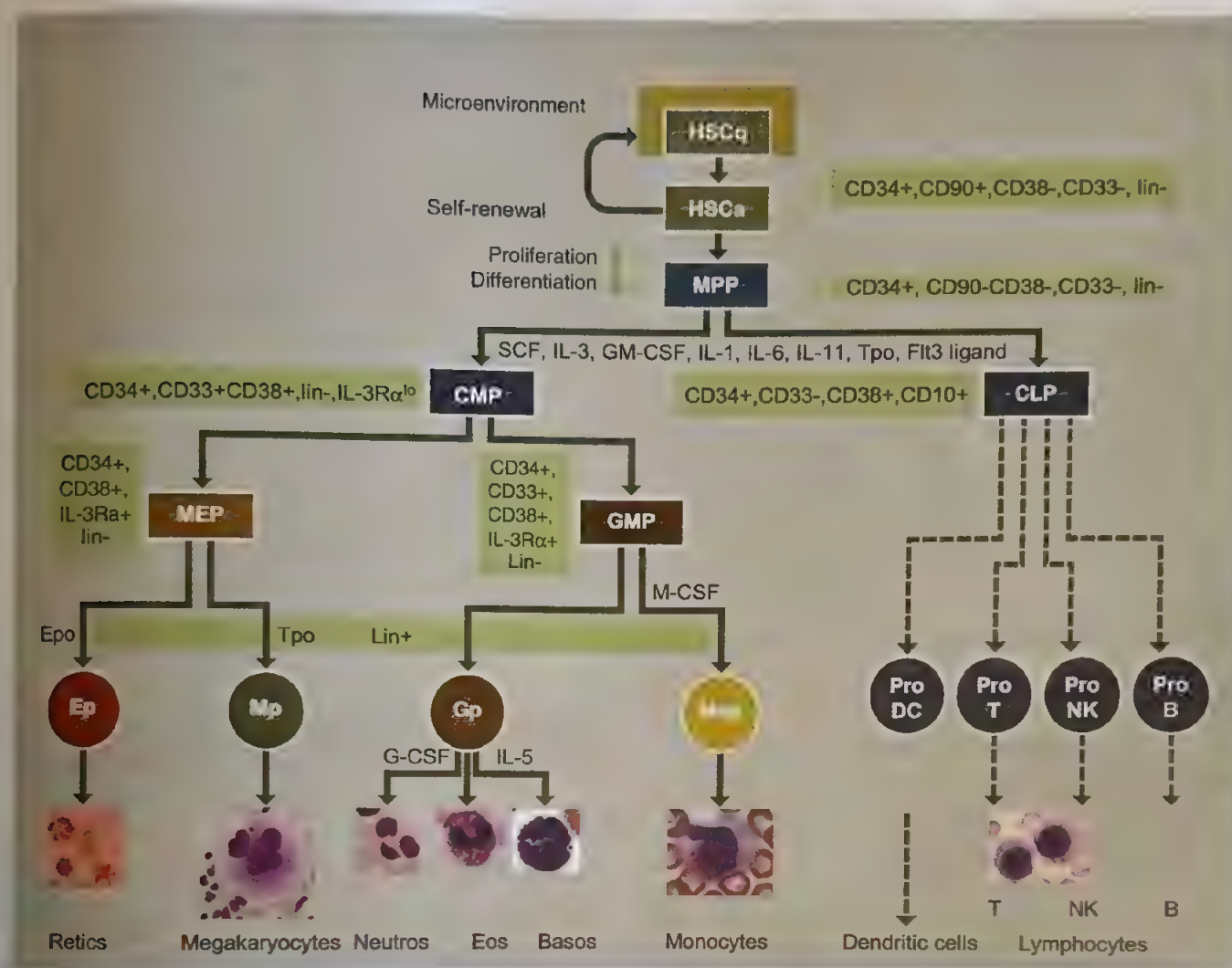


FIGURE 1-61 Abbreviated regulation of hematopoiesis by cytokines (not a comprehensive list of cytokines) with commonly known CD surface markers listed (not a complete list). There are two types of haemopoietic stem cells (HSC) reported in the bone marrow microvasculature; Long Term HSC (LT-HSC) and Short Term HSC (ST-HSC). The pluripotent HSCs differentiate into a multipotent progenitor stem cell (MPP) which differentiates into a common myeloid progenitor (CMP) and a common lymphoid progenitor (CLP). In the presence of hematopoietic cytokines and specific transcription factors, these progenitors differentiate and are committed to a single or bilineage progenitor that terminally differentiates into the mature blood cells. The CMP can differentiate into a megakaryocyte-erythroid progenitor (MEP) or a granulocyte monocyte progenitor (GMP). The MEP differentiates into the erythroid progenitor (Ep) and megakaryocyte progenitor (Mp). The GMP differentiates into the granulocyte progenitor (Gp) and monocyte progenitor (Mop). The CLP differentiates into B lymphocyte progenitor (pro B or Bp), T lymphocyte progenitor (Pro T or Tp), NK lymphocyte progenitor (Pro NK or NKp), and dendritic cell (Pro DC or DCp). HSCq = Long-term HSC (LT-HSC); HSCa = Short-term HSC (ST-HSC); IL = interleukin; EPO = erythropoietin; G-CSF = granulocyte colony-stimulating factor; M-CSF = macrophage-colony-stimulating factor; GM-CSF = granulocyte macrophage colony-stimulating factor; TPO = thrombopoietin; SCF = stem cell factor; Retics = reticulocytes; Neutros = neutrophils; Eos = eosinophils; Basos = basophils; T = t-lymphocytes; B = b-lymphocytes; NK = natural killer lymphocyte. Adapted from Patel A, Radia D. Haemopoiesis and the formation of blood cells. Elsevier Ltd. Clinical Sciences. Medicine 2017;45(4):194-197.

before the interactions between hematopoietic growth factors in the marrow microenvironment can be completely understood.

Clusters of Differentiation Nomenclature

Monoclonal antibody (mAb) technology and the development of multiple commercial sources under a variety of trade names and designations led to the development of a standardized nomenclature for human leukocyte differentiation antigens termed the *Clusters of Differentiation (CD) nomenclature*.

CD nomenclature is fundamentally utilized by the World Health Organization and the scientific community at large.⁶³ It provides a unified designation system for mAbs and the cell surface molecules that they recognize on leukocytes. CD molecules are commonly used as cell markers, allowing for the identification and isolation of leukocyte populations, subsets, and differentiation stages.⁶³ This nomenclature was determined by a series of international workshops on human leukocyte differentiation antigens (HLDA) by evaluating monoclonal antibodies produced in many laboratories with similar reactive patterns with tissue, cells, or molecules and compared.⁶³

TABLE 1-17 Cytokines Involved in Hematopoietic Blood Cell Development

Growth Factors	Synonym	Source	Target Cells Stimulated	Major Function
Epo	Erythropoietin	Kidney, liver	Erythroid progenitors	Stimulates formation of erythrocytes
G-CSF	Granulocyte colony-stimulating factor (CSF 3)	Macrophages, endothelial cells, fibroblasts	Stem cells, neutrophil precursors	Stimulates granulocyte production, mobilizes stem cells
GM-CSF	Granulocyte-macrophage-colony-stimulating factor (CSF2)	T lymphocytes, macrophages, endothelial cells, fibroblasts	Progenitors for neutrophils, eosinophils, monocytes	Multilineage hematopoietic growth factor, especially monocytes, neutrophils, eosinophils, and basophils
M-CSF	Monocyte-macrophage CSF (CSF-1)	Endothelial cells, fibroblasts, B cells, monocytes-macrophages, stromal cells	Mononuclear phagocytes	Stimulates macrophage colony formation
SCF	Stem cell factor, (c-kit ligand)	Fibroblasts MSC	Stem cells	HSC maintenance
EPO	Erythropoietin			Stimulates formation of erythrocytes
TPO	Thrombopoietin	Hepatocyte	Stem Cells	Stimulates formation of megakaryocytes/platelets
Interleukins				
IL-1	Hematopoietic-1; response modulator	Macrophages, neutrophils, endothelial cells, epithelial cells	Mononuclear phagocytes progenitor cells	Not directly involved in blood cell development but functions in the inflammatory response
IL-2	T-cell growth factor	T lymphocytes, macrophages	T cells, B cells	Immune response, T-cell differentiation
IL-3	Multi-CSF	T lymphocytes	Precursors of neutrophils, platelets, monocytes, eosinophils, basophils, stem cells	Multilineage hematopoietic growth factor
IL-4	B-cell stimulatory factor I	T lymphocytes	B cells, mast cells, T cells	T Helper cell differentiation
IL-5	B-cell growth factor II, eosinophil differentiation factor	T lymphocytes	B cells, granulocytes, monocytes, eosinophils	B-cell development, differentiation and function of myeloid cells; eosinophil activity
IL-6	Interferon β hybridoma growth factor	T lymphocytes, macrophages	Stem cells, B lymphocytes	hematopoiesis, lymphoid differentiation
IL-7	Lymphopoietin-1	Stromal cells, osteoblasts	Pre-B lymphocytes and T lymphocytes, early granulocytes	T-, B-cell growth factor
IL-8	Granulocyte chemotactic factor	Monocytes, T cells, fibroblasts	Neutrophils, T cells, basophils	Neutrophil chemotaxis
IL-9	T-cell growth factor III	T cells	BFU-E, T cells, mast cells	Stimulates T, B, and NK cells
IL-10	Cytokine synthesis inhibitory factor	T cells, macrophages, B cells,	B cells macrophage, T cells, mast cells	Anti-inflammatory, inhibits macrophage activation
IL-11	Adipogenesis inhibitory factor	Stromal, fibroblasts	Megakaryocyte, B cells, mast cells	Hematopoiesis (also involved in osteoblast differentiation)
IL-12	NK cell stimulatory factor	B cells, macrophages	T cells, NK cells	Stimulates T and NK cells
IL-14	High molecular weight B-cell growth factor	T cells	Activated B cells	induces B-cell proliferation

TABLE 1-17 Cytokines Involved in Hematopoietic Blood Cell Development—cont'd

Growth Factors	Synonym	Source	Target Cells Stimulated	Major Function
IL-15		Macrophages, monocytes, dendritic cells, bone marrow stromal cells, and various epithelial cells	NK and T lymphocytes	Stimulates T and NK cells
IL-21		T and NK lymphocytes	Lymphoid cells and macrophages,	Stimulates T, B, and NK cells
IL-31		T lymphocytes	Epithelial cells	Not directly involved in blood cell development but functions in the inflammatory response and cell-mediated immunity
IFN- γ	Interferon gamma	T lymphocytes	NK, T and B lymphocytes, epithelia cells, macrophages	Impaired HSC reconstitution capacity Impaired HSC maintenance
TNF- α	Tumor necrosis factor alpha	T lymphocytes, NK lymphocytes and macrophages	Myeloid cells Nucleated cells	Myeloid differentiation

BFU-E = burst-forming unit-erythroid; NK = natural killer lymphocyte; CXCL = chemokine ligand; HSC = hematopoietic stem cell; MSC = mesenchymal stem cells.

BOX 1-4 Groups of Contrasting Cytokines

- TNF-alpha and related molecules
- IL-1 family members
- TGF- β
- Factors Signaling Through Tyrosine Kinase Receptor such as M-CSF
- Chemokines
- # Cytokines Signaling Through The JAK/STAT Pathway

Note: Represents the largest group comprising hematopoietic growth factors (i.e., EPO), immunomodulatory cytokines (i.e., IL-2), and inflammatory cytokines (i.e., IFN γ). TNF = tumor necrosis factor, IL = Interleukin, TGF = transforming growth factor β , M-CSF = Macrophage colony-stimulating factor (originally described as a growth factor of the mononuclear phagocytic lineage)

These similar reacting antibodies were assigned to a “Cluster of Differentiation” and given a CD number for the antigen it reacts with. CD numbers with a lowercase “w” stands for “workshop” indicating a provisional cluster that may or may not be promoted to full CD status at subsequent workshops.⁶³ The designation of new CDs requires submission to the workshop of at least two independent mAbs that recognize the same molecule and present an identical pattern of reactivity.⁶⁴ CD nomenclature frequently uses a “+” symbol to indicate a CD number presence, and a “-” symbol attributed to absence, such as CD2⁺, CD4⁺, CD8⁺.⁶⁵ Now, more than 400 CD antigens have been classified by these workshops.⁶⁰ The tenth HLDA workshop was held in Australia in 2014.⁶⁵ The CD antibodies that define these leukocyte antigens are widely used in research, differential diagnosis, monitoring, and treatment of disease.⁶³ The HLDA workshops are now conducted by the Human

Cell Differentiation Molecules (HCDM) organization.⁶⁵ The HCDM names and characterizes CD molecules.

The current CD antigens for each cell lineage as determined by the HLDA Workshops can be found at www.hcdm.org.

ADVANCED CONTENT

Clinical Applications of Cell Surface Markers

The use of monoclonal antibodies has been recognized as an invaluable tool for the treatment of several malignancies and autoimmune diseases.⁶³ They are specific to cell surface markers (CDs) that allow phenotypic characterization of cells in disease states.⁴¹ By using flow cytometry (see Chap. 34), cells labeled with monoclonal antibodies are sorted and enumerated to identify a specific population of cells.⁶⁵

Certain cell markers have been identified as being present on the cell surface in disease states such as the acute leukemias, autoimmune disease, and thromboembolic disease. Cell markers have also been identified in the management of renal, cardiac, and bone marrow transplantation. Although diagnosis of disease states is dependent on clinical presentation, cytochemistry, and examination of morphology, flow-cytometry characterization of cells has added another dimension to disease classification.⁶⁶ Monoclonal antibodies are used to characterize cells in the acute leukemias. Such markers allow for the differentiation of myeloblasts, lymphoblasts, monoblasts, megakaryoblasts, and erythroid ontogeny.

CRITICAL THINKING QUESTION

1-4 What is the importance of identifying cell surface markers?

SUMMARY CHART

- Blood is composed of 55% plasma, the liquid portion, and 45% cells, the formed elements (RBCs, WBCs, and platelets).
- The average blood volume in an adult is 4 to 6 L.
- Plasma contains mainly water (91.5%), proteins (7%), other solutes (1.5%).
- The plasma proteins are albumin, globulin, and fibrinogen.
- Erythrocyte morphology is evaluated in the thin area of every stained smear where red cells are evenly distributed, do not overlap, and are close together.
- Platelets should be evaluated in the same thin area of every stained blood smear where red cells are described by counting the number of platelets in 10 or more oil immersion fields. The normal finding is 7 to 15 platelets per oil-immersion field.
- Blood smears should be well made and well stained to properly differentiate leukocytes, platelets, and erythrocytes.
- Normal neutrophil segmented cells contain from two to five lobes (usually three) connected by a threadlike filament(s) and constitute 50% to 70% of mature neutrophils in an adult.
- Normal neutrophil band cells have a horseshoe-shaped nucleus without evidence of a filament and make up only 2% to 6% of the blood cells in an adult.
- Normal eosinophil granules are large, round, and stain orange to reddish-orange; eosinophils are found in 0% to 4% of the blood cells in an adult.
- The majority of lymphocytes on an adult blood smear are small, have a relatively round nucleus with clumped chromatin and a small amount of pale blue cytoplasm; lymphocytes comprise 20% to 44% of the blood cells in an adult.
- A monocyte is larger than the mature neutrophil; has abundant gray blue cytoplasm with fine, reddish or purplish, evenly distributed granules; and has a nucleus with folds or brainlike convolutions, and lacy, often delicate, chromatin. Monocytes comprise 2% to 9% of blood cells in an adult.
- Large lymphocytes may reveal a few well defined purplish-red granules that can be easily counted, whereas numerous fine indistinct granules that cannot be enumerated are present in a monocyte.
- Hematopoiesis is defined as the dynamic processes of production and development of the various blood and marrow cells.
- Hematopoiesis begins in the mesoderm of the yolk sac and, after 2 months, migrates to the liver and spleen, where it remains until the seventh month, before finally shifting to the bone marrow, which becomes the major site of blood cell development in the fetus and after birth.
- Erythropoiesis identifies the entire process by which erythrocytes are produced in the marrow and develop from rubriblasts to diffusely basophilic cells and finally into a mature erythrocyte.
- Myelopoiesis refers to the production, proliferation, differentiation, division, storage, and delivery to the blood of neutrophils, eosinophils, basophils, and monopoiesis.
- A rubriblast differs little from a myeloblast: both have a round primitive nucleus, visible nucleoli, and chromatin strands that are distinct and dispersed; however, the rubriblast is slightly larger than a myeloblast and has more cytoplasm, which stains a deeper blue.
- A metarubricyte is recognized by the solid, blue-black, degenerated nucleus with nonlinear clumped chromatin and cytoplasm that is predominately pinkish because of increasing hemoglobin synthesis; however, there may remain a minimal amount of bluish cytoplasm due to RNA.
- A promyelocyte has dark blue granules throughout the cytoplasm and sometimes lying over the nucleus, a large nucleus with slightly condensed chromatin, and often faintly visible nucleoli.
- A neutrophilic myelocyte is identified by a round or oval nucleus with condensed and unevenly stained chromatin strands and secondary pinkish-staining (neutrophilic) granules.
- A neutrophilic metamyelocyte has a bean-shaped nucleus with the indentation less than half the width of the arbitrary round nucleus, noticeable chromatin clumping, and cytoplasm filled with pinkish (neutrophilic) secondary granules.
- Lymphoblasts contain a large, round nucleus with thin, evenly stained, nonclumped chromatin strands; one or more nucleoli; and a small amount of blue cytoplasm.
- Monoblasts are large and demonstrate a large nucleus (sometimes minimally indented), one or two prominent nucleoli, fine lacy nuclear chromatin, and nongranular, often deep blue cytoplasm.
- Plasmacytes are characterized by an eccentrically placed, round, small nucleus with lumpy chromatin; nongranular deep or vibrant blue cytoplasm; perinuclear clear area; occasional vacuoles; and slightly irregular margins.
- The mature megakaryocyte, the largest of the hematopoietic cells (range is 30 to 100 μm) in the bone marrow, has a multilobulated nucleus with coarse linear chromatin and bluish cytoplasm, containing numerous small, dense, reddish-blue granules, which fragment to form platelets.

CASE STUDY 1-1

A patient presents to their physician feeling tired and weak with no expected cause. Upon physical examination, they are found to be pale and have a lower than normal heart rate. Labs are drawn. The RBC count, Hgb, and Hct are all low.

Analysis	Result
RBC count	$3.1 \times 10^{12}/L$
Hgb	9.0 g/dL
Hct	27%

QUESTIONS

1. What is the patient's likely condition?
2. What cells might be observed on peripheral smear?

3. If evaluated, which cytokine would likely be increased in this patient?

ANSWERS

1. Anemia is likely due to low RBCs present.
2. In addition to lower numbers of RBCs, with anemia, an increased number of polychromatophilic cells (reticulocytes) are delivered early from the marrow to compensate for decreased in RBC concentration.
3. With anemia, erythropoietin stimulates marrow erythroid precursors to proliferate and increase the number of early erythroid cells.

CASE STUDY 1-2

A young male is admitted to the hospital with high fever and malaise. Upon admission, a blood culture and CBC is drawn for analysis. Gram stain reveals gram-positive bacteria in the blood.

QUESTIONS

1. Which leukocyte would you expect to be increased in the CBC results?
2. Would you expect to see any changes in maturation level of the cell population?
3. What would cause the presence of less mature cells, if found?

ANSWERS

1. Neutrophils are the body's first line of defense against bacterial infections.
2. It is likely that, due to an active infection, band neutrophils would also be present.
3. During an active infection, neutrophils can be in high demand as the immune system calls for their help in fighting bacteria. The bone marrow would be stimulated by cytokines to increase production of neutrophils, which could lead to less than fully mature segmented neutrophils being released into the bloodstream.

REVIEW QUESTIONS

1. Which organ is the primary site of hematopoiesis in the fetus from the second month to the seventh month of gestation?
 - a. Liver
 - b. Spleen
 - c. Bone marrow
 - d. Femur
2. Which listing represents the proper cell sequence of erythropoiesis?
 - a. Basophilic normoblast, pronormoblast, polychromatophilic normoblast, orthochromatic normoblast, reticulocyte, erythrocyte
 - b. Pronormoblast, basophilic normoblast, polychromatophilic normoblast, orthochromatic normoblast, reticulocyte, erythrocyte
 - c. Pronormoblast, basophilic normoblast, orthochromatic normoblast, polychromatophilic normoblast, reticulocyte, erythrocyte
 - d. Basophilic normoblast, pronormoblast, orthochromatic normoblast, polychromatophilic normoblast, reticulocyte, erythrocyte

Continued

REVIEW QUESTIONS—cont'd

3. What is the best description of an orthochromic normoblast?
 - a. Small, blue-black pyknotic nucleus; no nucleoli; pink cytoplasm
 - b. Round nucleus with visible nucleoli; indistinct and dispersed chromatin; blue cytoplasm
 - c. Coarse chromatin, ill-defined or absent nucleoli, predominantly blue cytoplasm with pink tinge
 - d. Small nucleus, thick and condensed nuclear chromatin, no nucleoli; mixture of pink and blue cytoplasm
4. What is the sequence for the maturation pools of granulocyte production?
 - a. Maturation, proliferation, storage, functional (or marginated) pool
 - b. Proliferation, maturation, storage, functional (or marginated) pool
 - c. Storage, maturation, proliferation, functional (or marginated) pool
 - d. Functional (or marginated) pool, storage, proliferation, maturation
5. Which listing represents the proper cell sequence of granulocytopoiesis?
 - a. Myeloblast, myelocyte, promyelocyte, metamyelocyte, band, segmented cell
 - b. Myeloblast, metamyelocyte, myelocyte, promyelocyte, segmented cell, band
 - c. Myeloblast, promyelocyte, myelocyte, metamyelocyte, band, segmented cell
 - d. Myeloblast, band, promyelocyte, myelocyte, metamyelocyte, segmented cell
6. Which granulocytic cell has a kidney-shaped nucleus with clumped chromatin and small, pink, secondary granules with a few primary dark granules?
 - a. Band
 - b. Myelocyte
 - c. Promyelocyte
 - d. Metamyelocyte
7. Which granulocytic cell has large, abundant violet-blue or purple-black granules?
 - a. Eosinophil
 - b. Basophil
 - c. Neutrophil
 - d. Monocyte
8. What is the proper cell sequence for the monocyte-macrophage phagocytic system?
 - a. Monoblast, macrophage, promonocyte, monocyte
 - b. Monoblast, monocyte, promonocyte, macrophage
 - c. Monoblast, promonocyte, monocyte, macrophage
 - d. Monoblast, promonocyte, macrophage, monocyte
9. Which cell classification is described by the following statements: second most numerous cell in the blood; usually small and round, intensely blue cytoplasm, and nucleus with clumped dark purple chromatin?
 - a. Monocyte
 - b. Lymphocyte
 - c. Null cell
 - d. Plasmacyte
10. What is the average blood volume?
 - a. 5 to 6 L
 - b. 4 to 6 L
 - c. 6 to 7 L
 - d. 3 to 5 L
11. What percent of the blood volume represents the formed elements?
 - a. 55%
 - b. 50%
 - c. 60%
 - d. 45%
12. Which of the following cells is the second most numerous cell in the blood comprising from 20% to 44% of the adult blood cells?
 - a. Lymphocyte
 - b. Platelet
 - c. Eosinophil
 - d. Monocyte
13. Which of the following describes a difference between a neutrophilic band and a neutrophilic segmented cell?
 - a. Band neutrophils have primary granules instead of secondary granules.
 - b. Band neutrophils lack a distinguishable filament between nuclear lobes.
 - c. Segmented neutrophils contain clear to pink cytoplasm where band neutrophils do not.
 - d. Band neutrophils have a round or oval nucleus shape.
14. Large lymphocytes are often difficult to distinguish between which other leukocyte?
 - a. Monocyte
 - b. Neutrophil
 - c. Osteoclast
 - d. Eosinophil
15. Which of the following characteristics can be MOST helpful when distinguishing a cell as a myeloblast?
 - a. The cytoplasm is dark blue in appearance.
 - b. The nuclear chromatin is smooth in composition.
 - c. There are vacuoles in the cytoplasm.
 - d. The nucleus can often contain 1 to 3 nucleoli

REVIEW QUESTIONS—cont'd

16. Which cell has a round eccentric nucleus, dark blue, abundant cytoplasm, with a perinuclear clear zone?
 - a. Neutrophil
 - b. Osteoblast
 - c. Plasmacyte
 - d. Platelet
17. Which cytokine is responsible for stimulating the production of red blood cells?
 - a. Thrombopoietin
 - b. Erythropoietin
 - c. IL-1
 - d. IL-4

See answers at the back of this book.

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The Red Blood Cell

Structure and Function

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CHAPTER OUTLINE

The Red Blood Cell Membrane

Red Blood Cell Membrane Proteins
Deformability
Permeability
Red Blood Cell Membrane Lipids

Hemoglobin Structure and Function

Hemoglobin Synthesis
Hemoglobin Function

Abnormal Hemoglobins of Clinical Importance

Maintenance of Hemoglobin Function:
Active Red Blood Cell Metabolic Pathways

Erythrocyte Senescence and Hemolysis

Extravascular Hemolysis
Intravascular Hemolysis

Summary Chart

Case Study 2-1

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 2-1 Describe the trilaminar membrane structure of the red blood cell, including the lipid and protein makeup of each layer.
- 2-2 Explain the functionality of glycophorin and spectrin.
- 2-3 Explain the importance of red blood cell membrane deformability and permeability.
- 2-4 Name the three processes required for normal hemoglobin production.
- 2-5 Evaluate normal adult hemoglobins and the globin chains found within each.
- 2-6 Describe the primary function of hemoglobin.
- 2-7 Define "shift to the right" in relation to the hemoglobin-oxygen dissociation curve.

- 2-8 Define "shift to the left" in relation to the hemoglobin-oxygen dissociation curve.
- 2-9 Identify abnormal hemoglobins and the conditions that can cause each.
- 2-10 Name the functions of red blood cell survival that require energy.
- 2-11 Name the most important metabolic pathways of the red blood cell.
- 2-12 Explain the role and process of the reticuloendothelial system (RES) in red blood cell senescence.
- 2-13 Contrast extravascular hemolysis with intravascular hemolysis.

Three areas of red blood cell (RBC) structure and metabolism are crucial for normal erythrocyte survival and function: the RBC membrane, hemoglobin structure and function, and metabolic pathways. Defects or problems associated with any of these areas will result in impaired RBC survival. A thorough working knowledge of these areas of RBC physiology will ensure basic understanding of the various complex erythrocyte functions. This chapter will outline and describe these areas so a deeper understanding of these important RBC aspects can be developed and applied to red blood cell pathologies presented in other chapters.

The Red Blood Cell Membrane

The RBC membrane viewed by transmission electron microscopy (TEM) appears as a trilaminar structure consisting of a

dark-light-dark band arrangement of layers (Fig. 2-1). These layers represent:

1. An outer hydrophilic portion chemically composed of glycolipid, glycoprotein, and protein
2. A central hydrophobic layer containing protein, cholesterol, and phospholipid
3. An inner hydrophilic layer containing protein

The RBC membrane is highly elastic, responds rapidly to applied stresses of fluid forces, and is capable of undergoing large membrane extensions without fragmentation.^{1,2}

The RBC membrane represents a highly complex structure consisting of a semipermeable lipid bilayer supported by a meshlike cytoskeleton structure² (Fig. 2-2). The RBC membrane cytoskeleton is a network of proteins on the inner surface

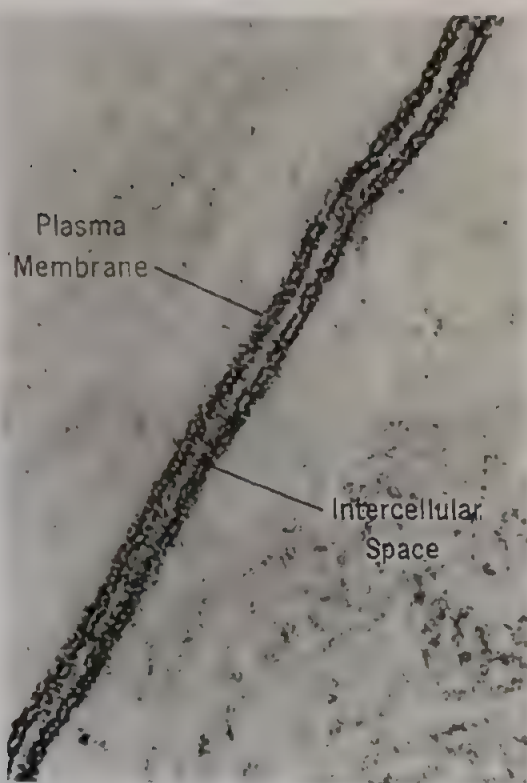


FIGURE 2-1 Transmission electron microscopy (TEM) of plasma membrane.

of the plasma membrane that is responsible for strengthening the lipid bilayer and maintaining the shape, stability, **deformability**, and flexibility of the RBC.^{1,2} The fluid lipid matrix contains equal amounts of cholesterol and phospholipids with a mosaic of proteins interspersed throughout at various intervals.³ The proteins that extend from the outer surface and traverse the entire membrane to the inner cytoplasmic side of the RBC are termed **integral membrane proteins**. The other major class of RBC membrane proteins, called **peripheral**

membrane proteins, is limited to the cytoplasmic surface of the membrane, which is beneath the lipid bilayer and forms the RBC cytoskeleton.⁴ Both the protein and the lipids are organized asymmetrically within the RBC membrane.³ The chemical composition of the membrane mass is approximately 40% lipids, 52% proteins, and 8% carbohydrates.¹

Red Blood Cell Membrane Proteins

In early studies, the RBC membrane proteins' nomenclature was based on their migration rate on sodium dodecyl sulfate (SDS) gels and labeled, for example, as bands 1 through 8. Today, with the use of the high-performance mass spectrometers and sophisticated data analysis methods (proteomics analysis), the red cell membrane contains nearly 20 major proteins and more than 2,000 minor proteins.⁵ These proteins have been categorized on the basis of their subcellular localization, protein family they belong to, and their function.^{4,5}

ADVANCED CONTENT

Of the membrane proteins identified, the percentage division of each protein is listed in Table 2-1. Many proteins are associated with the outer layer of the RBC membrane through a posttranslationally added glycosyl phosphatidylinositol (GPI) anchor.^{6,7} It is known that this linkage can increase lateral mobility of proteins migrating to the cell surface and reinserting into the RBC membrane.⁷ It has been postulated that this linkage may also be involved in cell signaling.⁷ The membrane-associated, GPI-anchored, and cytoskeletal proteins are considered "peripheral" proteins. Proteins have also been recently categorized according to molecular function and biological process.⁵ The majority of the proteins (34%) are involved in binding; others possess

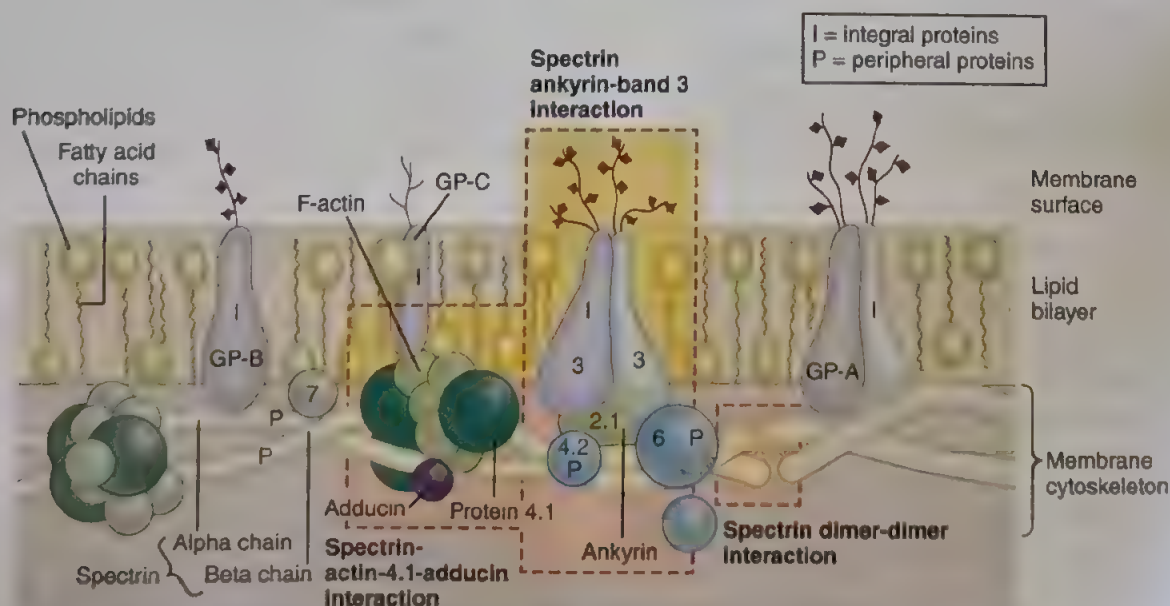


FIGURE 2-2 Schematic illustration of red blood cell membrane depicting the composition and arrangement of red cell membrane proteins. GP A = glycoprotein A; GP-B = glycoprotein B; GP-C = glycoprotein C. Numbers refer to pattern of migration of sodium dodecyl-polyacrylamide gel pattern stained with Coomassie brilliant blue. Relations of proteins to each other and to lipids are purely hypotheticalal positions of the proteins relative to the inside or outside of the lipid bilayer are accurate. (Note: Proteins are not drawn to scale and are omitted.)

TABLE 2-1 Subcellular Localization of Identified RBC Membrane Proteins

Membrane Protein	Percentage of RBC Membrane Proteins
Integral membrane proteins	31%
Membrane-associated or bound	16%
Cytoskeletal proteins	12%
Cytoplasmic proteins	12%
Organelle proteins	6%
GPI-anchored proteins	1%
Extracellular proteins	6%
Localization unavailable	16%

GPI = glycosyl phosphatidylinositol.

a catalytic activity (29%), 14% are transporter proteins, 9% are signal-transducer proteins, and 7% of the proteins are involved in structural activity.⁶

In terms of biological processes, 51% of the proteins identified are involved in cellular processes, 50% in physiological processes, and 11% in regulatory processes.⁵ Regulatory mechanisms in the RBC include regulation of cellular and physiological processes as well as enzyme activities. Because RBCs differentiate and mature during development, not all proteins are present in their active form.⁶ Degradation of organelles occurs during certain stages of maturation. In addition, during the 120-day life span of the mature RBC, chemical and enzymatic modifications occur.⁶

Two of the most important protein constituents of the RBC membrane are **glycophorin**, an integral membrane protein, and **spectrin**, a peripheral membrane protein. Table 2-2 lists selected integral and peripheral membrane proteins.^{6,7}

Glycophorin is the principal RBC glycoprotein, representing approximately 20% of the total membrane protein.⁶ The molecule contains approximately 60% carbohydrate and accounts for most of the membrane sialic acid, which gives the erythrocytes their negative charge. As a result, RBCs repel each other as they move through the circulation. Glycophorin, similar to other integral membrane proteins, spans the entire thickness of the lipid bilayer and appears on the external surface of the RBC membrane, accounting for the location of many RBC antigens.^{8,9} Most of these proteins carry RBC antigens and are receptors (such as the glycophorins) or transport proteins (such as the anion-exchange channel glycoprotein, Band 3). The anion-exchange channel comprises approximately 30% of the RBC membrane protein.⁴ The plasma membrane envelope is anchored to the RBC cytoskeleton network of proteins through tethering sites of integral proteins located in the lipid bilayer.⁴ The condensed fluid lipid bilayer plus integral proteins chemically isolates and regulates the cell interior. The RBC cytoskeleton network of proteins

TABLE 2-2 Red Cell Membrane Integral and Peripheral Proteins

Integral Proteins	Peripheral Proteins
Glycophorin A	Spectrin
Glycophorin B	Actin (band 5)
Glycophorin C	Ankyrin (band 2.1)
Glycophorin D	Band 4.1 and 4.2
Anion exchange-channel (Band 3)	Adducin

provides rigid support and stability to the lipid bilayer and is responsible for the deformability properties of the RBC membrane. It is speculated that the anion-exchange channel and the glycophorins play a major role in anchoring the RBC membrane cytoskeleton to the lipid bilayer.^{4,10} As a result, lateral mobility of these integral proteins within the lipid bilayer is relatively restricted.^{8,9}

The major components of the red cell cytoskeleton include spectrin, ankyrin, actin, protein 4.1, and other cytoskeletal proteins.⁴ Spectrin is clearly the most abundant peripheral protein of the RBC membrane cytoskeleton, comprising approximately 25% to 30% of the total membrane protein and 75% of the cytoskeleton.⁴

Spectrin is a long flexible wormlike protein composed of a helix of two parallel polypeptide chains, an alpha (α) chain (molecular weight 240,000 d) and a beta (β) chain (molecular weight 225,000 d).⁴ These chains are oriented in opposite directions and are intertwined side to side to form heterodimers. These chains link together with other $\alpha\beta$ chains to form $(\alpha\beta)_2$ tetramers.⁴ Each chain contains multiple repeats, with specialized functional domains at the "head" end for spectrin dimer-tetramer association and ankyrin binding.⁴ The domains at the "tail" end of spectrin are for binding to protein 4.1, protein 4.2, short filaments of actin, and other proteins.⁴ On average, approximately six spectrins bind per actin filament, leading to a pseudohexagonal arrangement.⁴ These multiprotein complexes at the ankyrin- and actin-binding ends of spectrin attach to the overlying lipid bilayer through integral protein band 3. The spectrin complexes tie the RBC cytoskeleton network together, stabilizing it.⁴

Spectrin is an important factor in RBC membrane integrity, because it binds with other peripheral proteins (actin, ankyrin, adducin, protein 4.1, protein 4.2) and other cytoskeletal proteins to form a skeletal network of microfilaments on the inner surface of the RBC membrane (see Fig. 2-2).⁶ These microfilaments strengthen the membrane, protecting the cell from being broken by circulatory shear forces, and also control the biconcave shape and deformability of the cell.¹⁰ In addition, the cytoskeletal network provides stability to the lipid bilayer interface.^{4,6}

The preservation of the spectrin complexes, and thus the integrity of the RBC membrane, requires phosphorylation of spectrin by a protein kinase present in the membrane, which is energy-dependent, being catalyzed by adenosine triphosphate (ATP).^{4,6}

As mentioned earlier, the normal chemical composition, structural arrangement, and molecular interactions of the erythrocyte membrane are crucial to normal RBC survival. In

addition, they play a critical role in two important RBC characteristics: deformability and permeability. Table 2-3 outlines characteristics of some major components of the red cell membrane.

CRITICAL THINKING QUESTION

2-1 What roles do the most important integral and peripheral RBC membrane proteins provide?

See answers to all Critical Thinking Questions at the back of this book.

Deformability

Three properties of the RBC membrane are crucial for **deformability**: cytoskeletal proteins, processes controlling intracellular ion and water handling, and membrane surface-to-volume ratio.^{10,11} RBC membrane deformability or flexibility is critical not only to RBC survival as the cell travels through the microvasculature but also for its function of oxygen delivery.^{11,12} Decreased cellular deformability and red cell shape changes have been recognized as distinguishing features of a number of congenital or hereditary hemolytic anemias leading to decreased RBC survival in these disorders (see Chapters 9, 10, and 11).^{10,11}

It has already been mentioned that a loss of ATP (energy) levels leads to a decrease in the phosphorylation of spectrin

and, in turn, to a loss of membrane deformability. An accumulation or increase in deposition of membrane calcium also results, causing an increase in membrane rigidity and loss of pliability.¹¹ These cells are at a marked disadvantage when they pass through the small (3- to 5- μ m diameter) sinusoidal orifices of the spleen, one of the functions of which is extravascular sequestration and removal of aged, damaged, or less deformable RBCs or fragments of their membranes (Fig. 2-3). The loss of RBC membrane is exemplified by the formation of **spherocytes** (Fig. 2-4), cells with a reduced surface-to-volume ratio, and the so-called **bite cells** (Fig. 2-5), in which the removal of a portion of membrane has left a permanent indentation in the remaining cell membrane. The survival time of these forms is also shortened.

Shape change from the normal symmetric and resilient discoid shape of the RBC can be stimulated by a variety of factors, which include both mechanically induced and chemically induced forces.¹³ Spectrin molecules exist in a folded conformation in the membrane of the nondeformed red cell. During reversible membrane deformability, certain spectrin molecules become uncoiled and extended, whereas others become more compressed and folded, resulting in a rearrangement of the cytoskeletal network.¹³ This reversible RBC membrane deformability results in a shape change while maintaining a constant surface area. The limit to reversible RBC

TABLE 2-3 Characteristics of Important Red Cell Membrane Proteins

Protein	Size	Function
Spectrin*	0.1- μ m heterodimeric filamentous "wormlike" protein consisting of a 240-kD α chain and a 225-kD β chain constitutes 25%–30% of the mass of membrane proteins	Principal structural element of RBC membrane, which plays a major role in the RBC cytoskeleton membrane organization
Ankyrin*	206-kD globular protein composed of 1879 amino acids	Attaches β -spectrin and the membrane skeleton to band 3 and to protein 4.2 in the ankyrin-linked band 3 complex
Adducin*	Heterodimer/tetramer of approximately 81 kD	Caps the end of actin and recruits spectrin to nearby sites on actin. Links actin junction to band 3.
Band 4.1*	Protein monomer of 66 kD	Binds to both β -spectrin and actin and greatly strengthens the spectrin-actin interaction. Links actin to the membrane via interactions with glycophorin and band 3. Competes with ankyrin for binding to band 3.
Band 3	Major RBC transmembrane protein and major integral protein that has two distinct domains: the transmembrane and cytoplasmic; Dimer or tetramer of 102 kD comprising 15%–20% of the total membrane protein	Anion-exchange channel forms membrane complexes with GPA, GPB, and other proteins that are linked to spectrin via ankyrin and protein 4.2, and linked to the actin junctional complex via protein 4.1, protein 4.2, contains binding sites for several glycolytic enzymes; plays a role in recognition and removal of normal senescent RBCs
Glycophorin (GP)	GPA, GPB	Binds band 3 in the ankyrin-linked band 3 complex; much of the red cell's negative surface charge attributed to GPA, GPB
	GPC, GPD	GPC and GPD link actin junctional complex to the RBC membrane via interactions with protein 4.1 and band 3
Band 4.2*	Thought to be a monomer of 78 kD	Binds to band 3 and ankyrin in the ankyrin-linked band 3 complex

*Denotes major components of the red cell cytoskeleton.

Source: Data from Lux IV SE. Anatomy of the red cell membrane skeleton. unanswered questions. Blood. 2016 Jan 14;127(2):187-199.

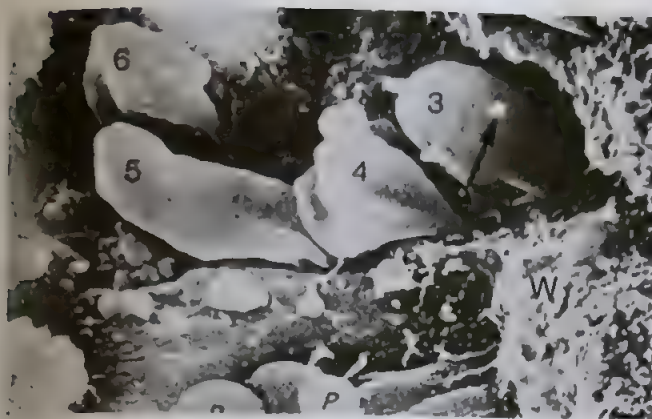


FIGURE 2-3 Scanning electron micrograph (SEM) of red cells (3 to 6) squeezing through fenestrated wall in transit from splenic cords to sinus. Epithelial linings of sinus wall to which platelets (P) adhere, along with "hairy" white blood cells (W), probably macrophages, are shown. (From Weiss, L. A scanning electron microscopic study of the spleen. *Blood*. 1974;43:665, with permission.)

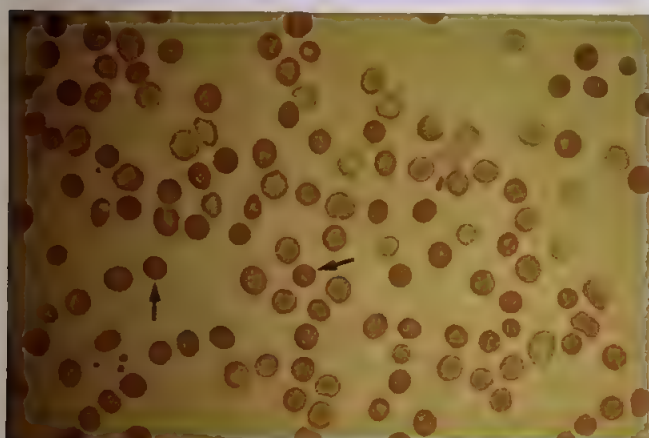


FIGURE 2-4 Spherocytes.

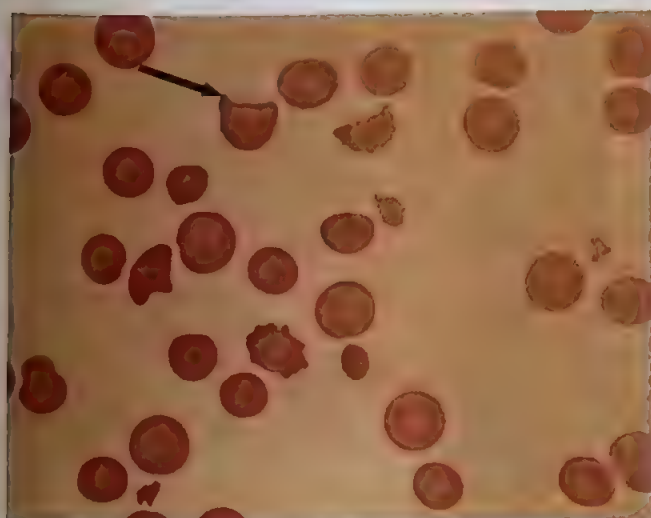


FIGURE 2-5 Bite cells.

membrane deformability occurs when applied forces break the protein-to-protein associations, necessitating an increase in surface area.¹⁴ RBC membrane deformability is characterized as irreversible when red cells are exposed to forces great enough to require an increase in surface area, leading to membrane fragmentation and instability of the cytoskeleton network.¹⁵

Permeability

The RBC membrane is freely permeable to water and anions; chloride (Cl^-) and bicarbonate (HCO_3^-) traverse the membrane in less than a second. It is speculated that this massive exchange of bicarbonate and chloride ions occurs through a large number of anion-exchange channels formed by the integral membrane proteins.¹⁶ In contrast, the RBC membrane is relatively impermeable to cations, with a half-time exchange of sodium (Na^+) and potassium (K^+) of more than 30 hours.¹⁶ It is primarily through the control of the sodium and potassium intracellular concentrations that the RBC maintains its volume and water homeostasis. Normally, K^+ is found primarily inside the red cell, and Na^+ is found outside the RBC. The erythrocyte intracellular-to-extracellular ratio for sodium is 1:12, and for potassium it is 25:1.¹⁶ The passive influx of sodium and potassium is controlled by as many as 300 cationic pumps that actively transport sodium out of the cell and potassium into the cell.¹⁶ The RBC ion leaks are balanced by two ATP-fueled pumps, the sodium and potassium pumps. Like other cationic pumps, these sodium-potassium pumps are energy-dependent, requiring ATP. The functional active transport of these particular cations by these cationic pumps also requires the membrane enzyme sodium-potassium ATPase. Calcium (Ca^{2+}) is also actively pumped from the interior of the RBC and into the plasma through the energy-dependent calcium-ATPase cationic pump, which is called the plasma membrane calcium pump (PMCA).¹⁶ **Calmodulin**, a cytoplasmic calcium-binding protein that contains four high-affinity calcium binding sites, is speculated to control these calcium-ATPase pumps.¹⁶ When calcium-calmodulin complexes form, the calcium-ATPase pump is activated, preventing excessive intracellular calcium buildup, which is deleterious to the RBC, resulting in shape changes and loss of deformability.¹⁶

The permeability properties of the RBC membrane, as well as active cation transport, are crucial to the prevention of colloid osmotic hemolysis and controlling the volume of the RBC.¹⁶ In addition, ATP-depleted cells allow the accumulation of excess intracellular calcium and sodium followed by potassium and water loss. This results in a dehydrated, rigid RBC that is subsequently sequestered by the spleen. Any abnormality that increases membrane permeability or alters cationic transport may lead to a decrease in RBC survival. In general, transport in RBCs is known to be diverse; it involves not only proteins, ions, and fluids but also carbohydrates, gases, and other substrates.¹⁶

CRITICAL THINKING QUESTION

2-2 Why is it important for the permeability of ions in and out of the RBC membrane to be regulated?

Red Blood Cell Membrane Lipids

Phospholipids

The erythrocyte membrane lipid consists of a bilayer of phospholipids interspersed with molecules of unesterified cholesterol that are present in nearly equimolar quantities.¹⁷ Free fatty acids and glycolipids are present in small quantities.¹⁸ Two groups of phospholipids, choline phospholipids and amino phospholipids, are known to possess a distinct asymmetry within the bilayer matrix of the RBC. The asymmetry allows selective movement of molecules into and out of the cell membrane.¹⁸

Choline phospholipids, consisting of phosphatidylcholine and sphingomyelin, are located primarily on the outside half of the lipid bilayer, readily accessible to the external environment.¹⁸ Because of their outward orientation in the lipid bilayer, the choline phospholipids may represent controlling points in the major pathways of lipid renewal, because there is an exchange between plasma fatty acids and the RBC membrane. Fatty acids are incorporated through an energy-dependent process into membrane phospholipids. Therefore, changes in body lipid transport and metabolism may cause abnormalities in the plasma phospholipid concentration that may alter the RBC membrane composition, resulting in decreased RBC survival in circulation. In addition, cholesterol has a pronounced effect on membrane fluidity.¹⁷ Membranes containing excess cholesterol are more viscous and less fluidic. This leads to a decrease in membrane deformability and RBC survival.

In contrast, amino phospholipids, consisting of phosphatidylethanolamine and phosphatidylserine, are located almost exclusively on the inside half or cytoplasmic side of the RBC membrane along with phosphatidylinositol.¹⁸ The specific orientation of these phospholipids maintains a precise lipid pattern that is critical to normal RBC survival in circulation. Alteration of this arrangement, leading to the abnormal appearance of these amino phospholipids on the outer surface of the lipid bilayer, promotes activation of the clotting cascade and may result in extravascular hemolysis.¹⁹ Stabilization of this phospholipid asymmetry in the erythrocyte membrane is maintained through the interaction with specific peripheral membrane proteins.¹⁸

Glycolipids and Cholesterol

Most of the glycolipids are located in the outer half of the lipid bilayer and interact with glycoproteins to form many of the RBC antigens.¹⁸ Cholesterol is approximately equally distributed, being located on both sides of the lipid bilayer inserted between the choline and amino phospholipids.¹⁷ Cholesterol comprises 25% of the RBC membrane lipid and is present in a 1:1 molar ratio with phospholipids.¹⁷ RBC membrane cholesterol is in continual exchange with plasma cholesterol and is, therefore, affected by changes in body lipid transport.¹⁷

As mentioned, accumulation of cholesterol results in a more viscous membrane with subsequent morphological changes in the RBCs such as target cells (Fig. 2-6) and may cause RBC membrane damage. **Acanthocytes**, RBCs with irregular, spiny projections called *spicules* (Fig. 2-7), have also been associated as a result of alterations in membrane lipids and proteins with various diseases.¹⁹

In summary, the majority of the RBC membrane is made of glycophorin and spectrin that are integral and peripheral

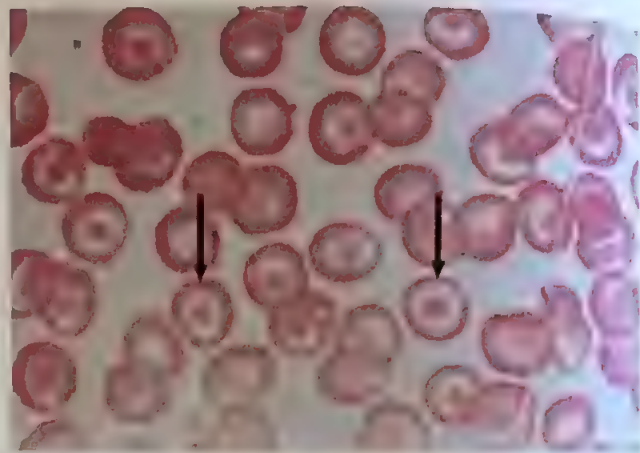


FIGURE 2-6 Target cells.

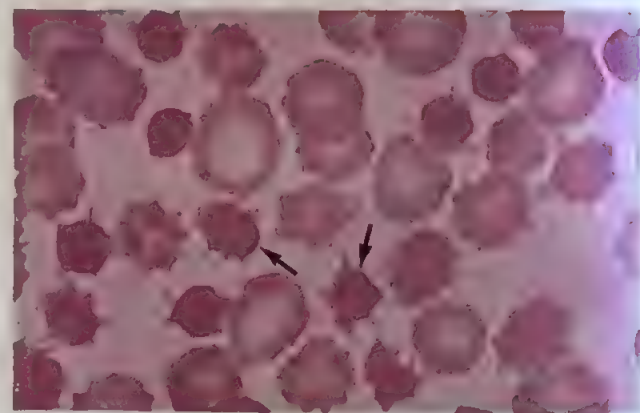


FIGURE 2-7 Acanthocytes.

proteins, respectively. Mutations in spectrin protein lead to hereditary elliptocytosis and hereditary spherocytosis.¹⁹ Any abnormality that increases membrane permeability or alters cationic transport may lead to a decrease in RBC survival. Accumulation of cholesterol results in more viscous cell membrane, which transforms the RBCs into target cells.

ADVANCED CONTENT

Acanthocytes may occur in patients with severe liver dysfunction, neuroacanthocytosis, abetalipoproteinemia, malnutrition, hypothyroidism, postsplenectomy, etc.²⁰ An excess accumulation of membrane cholesterol in association with liver disease and inherited lipid disorders such as abetalipoproteinemia and lecithin-cholesterol acyltransferase deficiency (LCAT) results in target cells or acanthocytes. The former disorder is caused by a decrease in apolipoprotein B (Apo B) in the blood with subsequent decreases in plasma and membrane lipids. The patient's red cells appear as acanthocytes with increased membrane rigidity. Target cell formation results from the subsequent increase in membrane cholesterol. All of these RBCs have a decreased survival rate because the excess lipid makes the cell membrane less deformable.

The abnormalities that can lead to a change in RBC morphology are summarized in Table 2-4.

TABLE 2-4 Abnormalities That Can Lead to a Change in RBC Morphology

Abnormality	RBC Morphology
Cholesterol accumulation in the RBC membrane (i.e., liver disease)	Target cells
Abetalipoproteinemia with cholesterol accumulation	Acanthocytes
LCAT deficiency with cholesterol accumulation	Hemolysis with red cell fragmentation
Decreased phosphorylated spectrin or altered spectrin	Bite cells and spherocytes

Hemoglobin Structure and Function

Hemoglobin, a conjugated globular protein with a molecular mass of approximately 64.4 kilodaltons (kD), constitutes 95% of the RBC dry weight, or 33% of the RBC weight by volume.²¹ Approximately 65% of the hemoglobin synthesis occurs during the nucleated stages of RBC maturation, and 35% occurs during the reticulocyte stage.²¹ Normal hemoglobin consists of globin (a tetramer of two pairs of globin polypeptide chains) and four heme groups, each of which contains a protoporphyrin ring plus ferrous iron (Fe^{2+}) (Fig. 2-8).

Hemoglobin Synthesis

Normal hemoglobin production is dependent on three processes (Fig. 2-9):

1. Adequate iron delivery and supply
2. Adequate synthesis of **protoporphyrins** (the precursor of heme)
3. Adequate globin synthesis

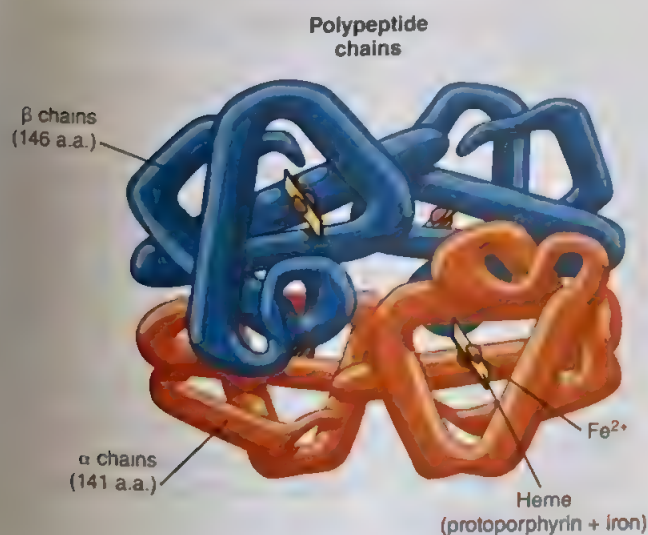


FIGURE 2-8 Hemoglobin A molecule comprised of two alpha, two beta, and four iron-containing heme groups. Beta chains have 146 amino acids and alpha chains have 141 amino acids.

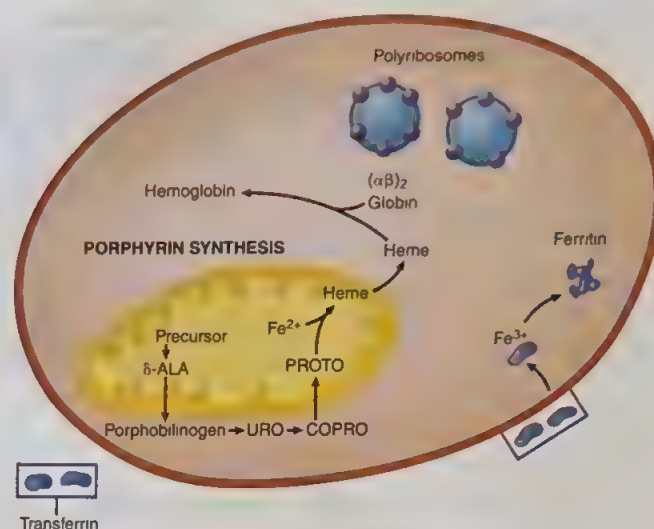


FIGURE 2-9 Hemoglobin synthesis in the reticulocyte. δ -ALA = delta-aminolevulinic acid; URO = uroporphyrinogen; COPRO = coproporphyrinogen; PROTO = protoporphyrin.

Iron Delivery and Supply

Iron, in the ferric state (Fe^{3+}), is delivered to the membrane of the RBC precursor by the protein carrier transferrin. Most of the iron that crosses the membrane and enters the cytoplasm of the cell is committed to hemoglobin synthesis and thereby proceeds to the mitochondria (where ferric iron is reduced to ferrous [Fe^{2+}] iron for insertion into the protoporphyrin ring to form heme).²² Excess iron in the cytoplasm aggregates as **ferritin**, the amount of which depends on the ratio between the level of plasma iron and the amount of iron required by the erythrocyte for hemoglobin synthesis. Two-thirds of the total body iron supply is bound to heme in the hemoglobin molecule (see Chapter 7 for a discussion of iron kinetics).

Synthesis of Protoporphyrins

Protoporphyrin synthesis begins in the mitochondria with the formation of delta-aminolevulinic acid (δ ALA) from the amino acid, glycine, and succinyl coenzyme A (CoA), which is the major rate-limiting step in heme biosynthesis (Fig. 2-10).²³ The mitochondrial enzyme, δ ALA synthetase, which mediates this reaction, is influenced by EPO and requires the presence of the cofactor pyridoxal phosphate (vitamin B_6).²³

Porphyrinogens, not porphyrins, are the intermediates of heme synthesis. Porphyrinogens are unstable tetrapyrroles that are readily and irreversibly oxidized to form **porphyrins**. In contrast, porphyrins are highly stable resonating molecules that are normally found in small quantities in the urine as a result of normal RBC catabolism.²⁴

Excessive formation of porphyrins can occur if any one of the normal enzymatic steps in heme synthesis is blocked and can result in one of several metabolic disorders, collectively called the *porphyrias*²⁴ (see Chapter 7).

Globin Synthesis

Globin chain synthesis occurs on RBC-specific cytoplasmic ribosomes, which are initiated from the inheritance of

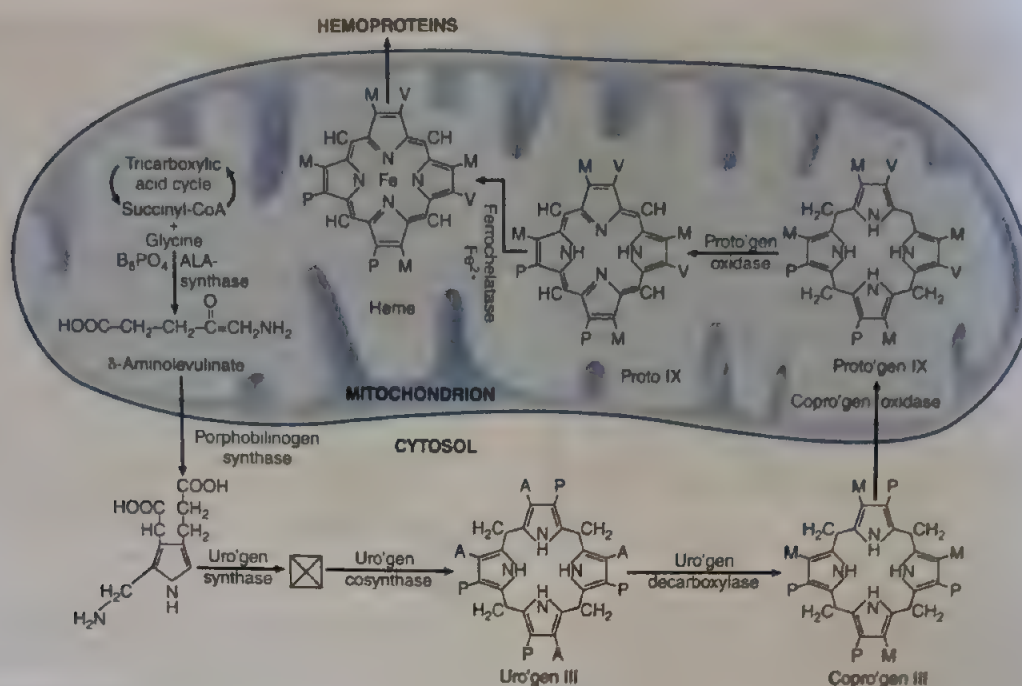


FIGURE 2-10 Synthesis of heme. The heme biosynthetic pathway, showing the distribution of enzymes between the mitochondria and the cytoplasm. Condensation of glycine and succinyl coenzyme A yields ALA, which is irreversible; two molecules of ALA undergo condensation by the enzyme ALA dehydratase to yield porphobilinogen (PBG). In the presence of uroporphyrinogen III cosynthase and uroporphyrinogen I synthase, PBG yields uroporphyrinogen III. Uroporphyrinogen III undergoes four decarboxylation steps, catalyzed by the enzyme uroporphyrinogen decarboxylase, to yield coproporphyrinogen III. Coproporphyrinogen III is transported from the cytosol into the mitochondria, where the enzyme coproporphyrinogen oxidase acts on the propionic acid side chains to yield protoporphyrinogen IX. Catalyzed by protoporphyrinogen IX oxidase, protoporphyrinogen IX is oxidized to protoporphyrin IX. Protoporphyrin IX combines with ferrous iron to yield heme (catalyzed by heme synthase). (Intermediates between uroporphyrinogen and coproporphyrinogen, designated by X, remain unidentified.) B_6PO_4 = pyridoxal phosphate. (From Tietz, MW. Textbook of Clinical Chemistry, Philadelphia: WB Saunders; 1986, with permission.)

various structural genes. Each gene results in the formation of a specific polypeptide chain. Each somatic diploid cell, including the RBC, contains four alpha (α), two zeta (ζ), two beta (β), two delta (δ), two epsilon (ϵ), and four gamma (γ) genes.²⁴ The α and ζ genes are located on chromosome 16, and the β , δ , ϵ , and γ genes on chromosome 11 (Fig. 2-11). The resulting gene products formed have been called α , ζ , β , δ , ϵ , and γ globin chains. Throughout embryonic and fetal development, activation of the globin genes progresses from the ζ to the α gene and from the ϵ to the γ , δ , and β genes.²⁴

The ϵ and ζ chains normally appear only during embryonic development (Table 2-5). These two chains, plus the α and γ chains, are constituents of embryonic hemoglobins: Hb Gower 1 ($\zeta_2\epsilon_2$), Hb Gower 2 ($\alpha_2\epsilon_2$), and Hb Portland ($\zeta_2\gamma_2$).²³ The ϵ and ζ chains are produced up to approximately 3 months after conception.²⁴ The α chain is always present. Production of γ chains is active from the third month of fetal development until 1 year postnatally.²⁴ In the fetus, the major hemoglobin is $\alpha_2\gamma_2$ (hemoglobin F). The γ chains occur as a mixture of two types of chains, differing only by one amino acid at position 136. G-gamma ($^G\gamma$) contains glycine, whereas A-gamma ($^A\gamma$) has alanine at that position.²⁴ The ratio of $^G\gamma$ to $^A\gamma$ is approximately 3:1 at birth and 2:3 by 1 year of age.²⁴ By the age of 2 years, hemoglobin F comprises less than 2% of the total hemoglobin. Production of β chains rises gradually prenatally and reaches adult percentages between 3 and 6 months postnatally.²⁴ Figure 2-12

depicts the time sequence of globin chain synthesis during fetal development, birth, and infancy.

All normal adult hemoglobins are formed as tetramers consisting of two α chains plus two (non- α) globin chains. Normal adult RBCs contain the following types of hemoglobin:

- 95% to 97% of the hemoglobin is HbA, which consists of $\alpha_2\beta_2$ chains
- 2% to 3% of the hemoglobin is HbA₂, which consists of $\alpha_2\delta_2$ chains
- 1% to 2% of the hemoglobin is HbF (fetal hemoglobin), which consists of $\alpha_2\gamma_2$ chains

In the cytoplasm, each synthesized globin chain links with heme (ferroprotoporphyrin IX) to form hemoglobin, which primarily consists of two α chains, two β chains, and four heme groups²³ (see Fig. 2-8). The structure of the chains and the resulting hemoglobin molecule is described in the following four steps:

1. The primary structure relates to the number and sequence of amino acids constituting each globin chain. The α chains have 141 amino acids, and the non- α chains have 146 amino acids. The sequence of amino acids is different in each chain.
2. The secondary structure occurs with the twisting of the amino acid chain around an axis in a helical conformation.

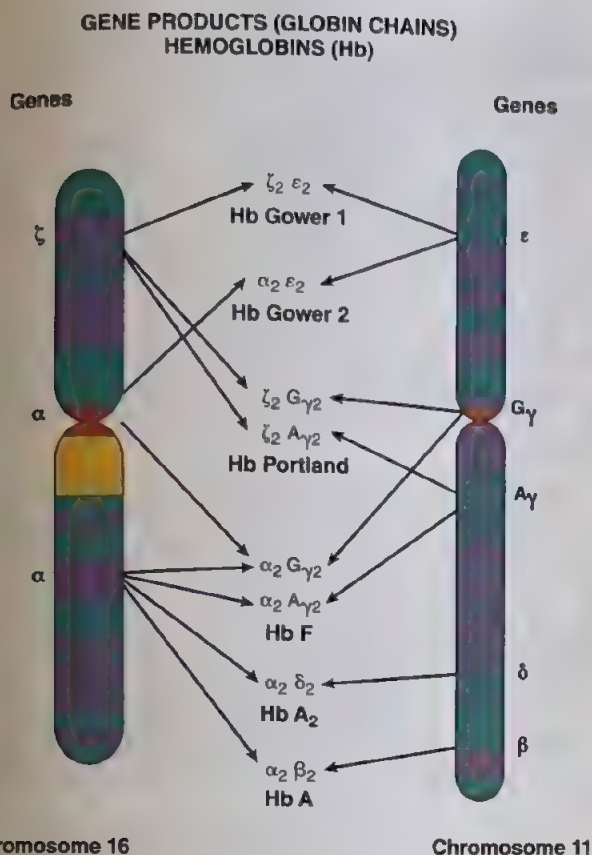


FIGURE 2-11 Genetic control and formation of human hemoglobins.

TABLE 2-5 Composition of Hemoglobin Found in Normal Human Development

Globin Chain	Hemoglobin	Stage of Development
$\alpha_2\epsilon_2$	Gower 2	Embryo
$\zeta_2\epsilon_2$	Gower 1	
$\zeta_2\gamma_2$	Portland	
$\alpha_2\gamma_2$	F	Fetus
$\alpha_2\gamma_2$	F	
$\alpha_2\beta_2$	A	Adult
$\alpha_2\delta_2$	A ₂	

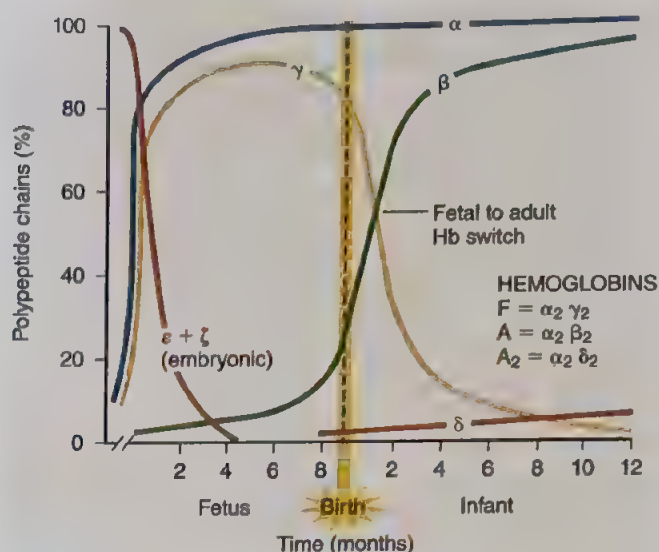


FIGURE 2-12 Changes in globin chain synthesis during fetal development, birth, and infancy.

Table 2-5 shows the composition of hemoglobin found during normal human development. The precise order of amino acids is critical to the structure and function of the hemoglobin molecule.

The rate of globin synthesis is directly related to the rate of porphyrin synthesis and vice versa: protoporphyrin synthesis is reduced when globin synthesis is impaired. There is, however, no such relationship with iron uptake when either globin or protoporphyrin synthesis is impaired; iron accumulates in the RBC cytoplasm as ferritin aggregates. The iron-laden, nucleated RBC is termed a *sideroblast* and the anucleated form, a *siderocyte*, when stained with Prussian blue for visualization of iron (Fig. 2-13).²⁵ When protoporphyrin synthesis is impaired, the mitochondria become encrusted with iron, which is visible around the nucleus of the RBC precursor when stained with Prussian blue. Such an RBC is termed a *ringed sideroblast* and is diagnostic for a pathogenesis linked to deficient protoporphyrin synthesis.²⁵

FIGURE 2-13 Ringed sideroblast and siderocytes as detected by Prussian blue staining of a bone marrow aspirate. (Prussian blue stain, $\times 1000$).

- The tertiary structure consists of bending the twisted amino acid chain into a three-dimensional shape resembling an "irregular pretzel." The polar groups are oriented outward, and the nonpolar groups are interior. The heme molecule is nestled in a nonpolar pocket and attached to a proximal histidine residue.
- The quaternary structure is the assembling of the four three-dimensional chains with their respected heme groups. The result is a complete, functional hemoglobin molecule (see Fig. 2-8).

Hemoglobin Function

The primary function of hemoglobin is delivery and release of oxygen to the tissues and facilitation of carbon dioxide excretion.¹² Because of hemoglobin's multichain structure, the molecule is capable of a considerable amount of allosteric movement as it loads and unloads oxygen.²³ One of the most important controls of hemoglobin affinity for oxygen is the RBC organic phosphate 2,3-diphosphoglycerate (2,3-DPG), now called 2,3-biphosphoglycerate (2,3-BPG). The unloading of oxygen by hemoglobin is accompanied by the widening of the space between β chains and the binding of 2,3-BPG on a mole-for-mole basis, with the formation of anionic salt bridges between the α d chains^{23,24} (Fig. 2-14). The resulting conformation of the **deoxyhemoglobin** molecule is known as the tense (T) form, which has a lower affinity for oxygen. When hemoglobin loads oxygen and becomes **oxyhemoglobin**, the established salt bridges are broken and α chains are pulled together, expelling 2,3-BPG. This relaxed form of the hemoglobin molecule has a higher affinity for oxygen. Each iron-containing heme group in each of the polypeptide chains of the hemoglobin molecule can bind one oxygen molecule.^{23,24}

These changes in shape that occur as the hemoglobin loads and unloads oxygen are referred to as the **respiratory movement**.²⁴ The dissociation and binding of oxygen by hemoglobin are not directly proportional to the oxygen tension (P_{O_2}) of its environment but instead exhibit a sigmoid-curve relationship known as the **hemoglobin-oxygen dissociation curve** depicted in Figure 2-14. The shape of this curve is very important physiologically because it permits a considerable amount of oxygen to be delivered to the tissues with a small drop in oxygen tension. For example, in the environment of the lungs, where the P_{O_2} measured in millimeters of mercury (mm Hg), is nearly 100 mm Hg, the hemoglobin molecule is almost 100% saturated with oxygen (see Fig. 2-14, point A). As the RBCs travel to the tissues, where the P_{O_2} drops to an

average 40 mm Hg (mean venous oxygen tension), the hemoglobin saturation drops to approximately 75%, releasing approximately 25% of the oxygen to the tissues (see Fig. 2-14, point B).

This is the normal situation of oxygen delivery at basal metabolic rate. In conditions such as hypoxia, a compensatory "**shift to the right**" of the hemoglobin-oxygen dissociation curve (Fig. 2-15) occurs to alleviate a tissue oxygen deficit. This rightward shift of the curve, mediated by increased levels of 2,3-BPG, results in a decrease in the affinity of hemoglobin for the oxygen molecule and an increase in oxygen delivery to the tissues.^{23,24} The RBCs therefore, have become more efficient in terms of oxygen delivery.

Therefore, a patient who is suffering from an anemia, caused by a loss of RBCs, may be able to compensate by shifting the oxygen dissociation curve to the right, making the RBCs, although fewer in number, more efficient. Some patients may be able to tolerate anemia better than others because of this compensatory mechanism. A shift to the right also may occur in response to acidosis or a rise in body temperature.²⁴ The shift to the right of the hemoglobin-oxygen dissociation curve is only one way in which patients may compensate for various types of hypoxia; other ways include increases in total cardiac output and in erythropoiesis.²⁴

A **shift to the left** of the hemoglobin-oxygen dissociation curve conversely results in an increase in hemoglobin-oxygen affinity and a decrease in oxygen delivery to the tissues (see Fig. 2-15). With such a dissociation curve, RBCs are much less efficient in releasing oxygen to the tissues. Among the conditions that can shift the oxygen dissociation curve to the left are alkalosis; decrease in body temperature; increased quantities of abnormal hemoglobins, such as methemoglobin and carboxyhemoglobin; increased quantities of hemoglobin F; or multiple transfusions of 2,3-BPG-depleted stored blood (attesting to the importance of 2,3-BPG in oxygen release).^{23,24}

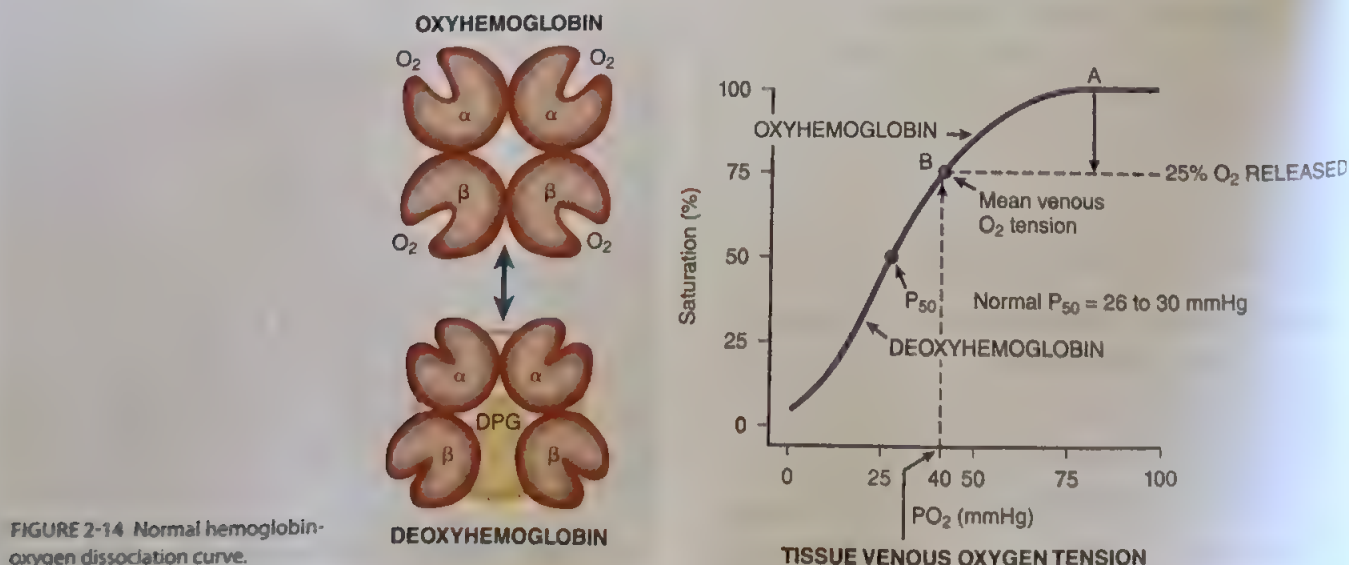


FIGURE 2-14 Normal hemoglobin-oxygen dissociation curve.

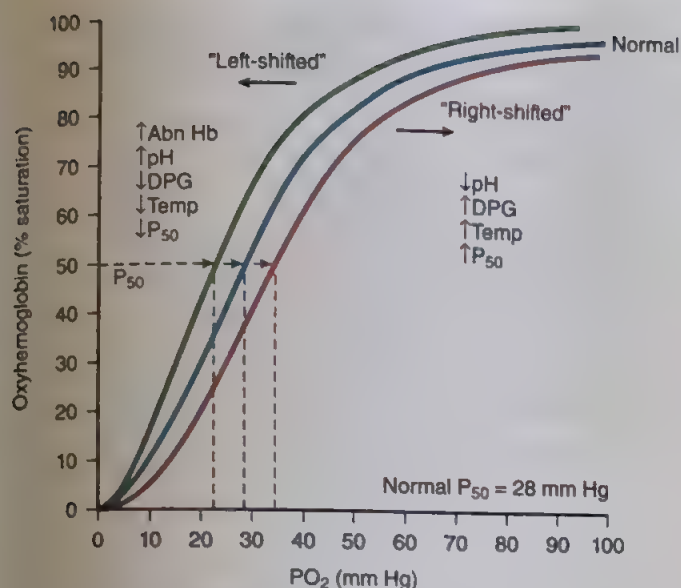


FIGURE 2-15 Hemoglobin-oxygen dissociation curve. Blue line is the normal curve. The green line represents a "shift to the left" that can occur with an increase in abnormal hemoglobins, increase in pH (alkalosis), a decrease in 2,3-DPG, and a decrease in body temperature. The red line represents a "shift to the right" that can occur with a decrease in pH (acidosis), an increase in 2,3-DPG, and an increase in body temperature.

ADVANCED CONTENT

Hemoglobin-oxygen affinity also can be expressed by P_{50} values, which designate the PO_2 at which hemoglobin is 50% saturated with oxygen under standard in vitro conditions of temperature and pH.²⁴ The P_{50} of normal blood is 26 to 30 mm Hg.²⁴ An increase in P_{50} represents a decrease in hemoglobin-oxygen affinity, or a shift to the right of the oxygen dissociation curve. A decrease in P_{50} represents an increase in the hemoglobin-oxygen affinity, or a shift to the left of the hemoglobin-oxygen dissociation curve. In addition to the reasons listed previously for shifts in the curve, inherited abnormalities of the hemoglobin molecule can result in either situation. These abnormalities are described by the P_{50} measurements. Abnormalities in hemoglobin structure or function can therefore have profound effects on the ability of RBCs to provide oxygen to the tissues.

Abnormal Hemoglobins of Clinical Importance

The hemoglobins previously described, oxyhemoglobin and reduced hemoglobin, are physiological hemoglobins because they function in the transport and delivery of oxygen within the circulation. Abnormal hemoglobins of clinical significance that are unable to transport or deliver oxygen include the following:

- Carboxyhemoglobin
- Methemoglobin
- Sulfhemoglobin

In **carboxyhemoglobin**, the oxygen molecules bound to heme have been replaced with carbon monoxide (CO). This

replacement process is relatively slow and dependent on the concentration of carbon monoxide in the blood. Once attached, however, the binding of carbon monoxide to the heme of the hemoglobin molecule is 200 times tighter than the binding of oxygen to heme.²⁶ The concentration of carbon monoxide can be increased in a number of conditions, including chronic heavy smoking. The toxic level of carboxyhemoglobin is 5.0 units.

Methemoglobin is formed when the iron of the hemoglobin molecule is oxidized to the ferric (Fe^{3+}) state.²⁴ Normally, less than 1% of the total circulating hemoglobin is in the methemoglobin form.²⁴ Increased formation of methemoglobin can occur as a result of an overload to oxidant stress, owing to the ingestion of strong oxidant drugs or an enzyme deficiency. The toxic level of methemoglobin is 1.5 units.

Sulfhemoglobin is formed when a certain situation or condition, such as ingestion of a sulfur-containing drug or chronic constipation, causes the sulfhemoglobin content of the blood to build up.²⁴ Sulfhemoglobin is incapable of carrying oxygen and represents an irreversible change of the hemoglobin molecule that persists until the RBCs are removed from the circulation. Both carboxyhemoglobin and methemoglobin, however, can be reverted to oxyhemoglobin through the use of oxygen inhalation and the administration of strong reducing substances, respectively.²⁴ The toxic level of sulfhemoglobin is 0.5 units.

At toxic levels of these hemoglobins, the tissue oxygen deficit results in cyanosis and anemia, and eventually death may occur.

CRITICAL THINKING QUESTION

2-3 How can a patient suffering with anemia still compensate for oxygen needs throughout the body?

Maintenance of Hemoglobin Function: Active Red Blood Cell Metabolic Pathways

Active erythrocyte metabolic pathways are necessary for the production of adequate ATP levels. Such generated energy is crucial to RBC survival and function in that it is necessary for maintaining:

- Hemoglobin function
- Membrane integrity and deformability
- RBC volume
- Adequate amounts of reduced pyridine nucleotides
- Protection of metabolic enzymes²⁴

RBCs generate energy almost exclusively through the anaerobic breakdown of glucose, because the metabolism of the anucleated erythrocyte is more limited than that of other body cells. The adult RBC possesses little ability to metabolize fatty acids and amino acids. In addition, mature RBCs contain no mitochondrial apparatus for oxidative metabolism (Table 2-6). The RBC metabolic pathways are mainly anaerobic, fortunately, because the function of the RBC is to deliver oxygen and not to consume it. Four pathways of RBC metabolism will be considered: the anaerobic glycolytic pathway and three ancillary pathways that serve to maintain the function of

TABLE 2-6 Comparison of Red Blood Cell Metabolic Activities During Various Stages of Maturation

Metabolic Activity	Nucleated RBC	Reticulocyte	Adult RBC
Replication	+	0	0
DNA synthesis	+	0	0
RNA synthesis	+	0	0
Lipid synthesis	+	+	0
RNA present	+	+	0
Heme synthesis	+	+	0
Protein synthesis	+	+	0
Mitochondria	+	+	0
Krebs tricarboxylic acid cycle	+	+	0
Embden-Meyerhof pathway	+	+	+
Pentose phosphate pathway	+	+	+
Maturation and/or senescence	+	+	+

hemoglobin (Fig. 2-16). All of these processes are essential if the RBC is to transport oxygen and maintain the physical characteristics required for its survival in circulation.

Of the ATP needed by RBCs, 90% is generated by the **Embden-Meyerhof glycolytic pathway**.²⁴ Here, the metabolism of glucose results in the net generation of two molecules of ATP. Although this ATP synthesis is inefficient compared with cells that use the Krebs cycle (aerobic metabolism), it provides sufficient ATP for the requirements of the RBCs. Glycolysis also generates NADH from NAD⁺, which is important in some of the other RBC metabolic pathways.

Another 5% to 10% of glucose is metabolized by the **hexose monophosphate (HMP) shunt** (also called the *phosphoglucomate pathway*).²⁴ This pathway produces the pyridine nucleotide NADPH from NADP⁺. NADPH, together with reduced glutathione, provides the main line of defense for the RBC against oxidative injury.²⁴ Oxidant drugs, as well as infections, can cause the accumulation of hydrogen peroxide and other oxidants, which can be toxic to cell proteins. The sequence of biochemical reactions shown in Figure 2-16 occurs within the normal RBC with adequate levels of appropriate enzymes and substrate to prevent the accumulation of these agents.

When the HMP shunt is functionally deficient, the amount of reduced glutathione becomes insufficient to neutralize intracellular oxidants. This results in globin denaturation and precipitation as aggregates (Heinz bodies) within the cell. If this process sufficiently damages the membrane, cell destruction occurs. Inherited defects in the pentose phosphate glutathione pathway, the most common of which is

glucose-6-phosphate dehydrogenase (G6PD) deficiency, result in the formation of Heinz bodies with subsequent microvascular hemolysis.²⁴ (Refer to Chapter 10, Enzyme Deficiencies.) Glutathione is not only crucial to keeping hemoglobin in a functional state, it is also important in maintaining its integrity by reducing sulfhydryl groups of hemoglobin, membrane protein, and enzymes subsequent to oxidation.

The **methemoglobin reductase pathway** is another important component of RBC metabolism. Two methemoglobin reductase systems are important in maintaining hemoglobin in the reduced (Fe^{2+} , ferrous) functional state.²⁴ Both pathways depend on the regeneration of reduced pyridine nucleotide and are referred to as the NADH and NADPH methemoglobin reductase pathways. In the absence of the enzyme methemoglobin reductase and the reducing action of the pyridine nucleotide NADH, there is an accumulation of methemoglobin, resulting from the conversion of the ferrous iron of heme to the ferric form (Fe^{3+}). Methemoglobin is a nonfunctional form of hemoglobin, having lost oxygen transport capabilities, as the metheme portion cannot combine with oxygen. Normal efficiency of the methemoglobin reductase pathway is exemplified by the fact that usually no more than 1% of RBC hemoglobin exists as methemoglobin in the RBCs of healthy individuals.²⁴

Another important pathway that is crucial to RBC function is the **Leubering-Rapaport shunt**. This pathway causes an extraordinary accumulation of the RBC organic phosphate 2,3-bisphosphoglycerate acid (2,3-BPG), which is important because of its profound effect on the affinity of hemoglobin for oxygen. Stores of this organic phosphate can serve as a reserve for additional ATP generation.

CRITICAL THINKING QUESTION

2-4 Why is RBC generation of energy mainly an anaerobic process?

Erythrocyte Senescence and Hemolysis

The RBC, a 7- to 8- μm biconcave disc (Fig. 2-17), travels 20 to 300 miles during its 120-day life span. During this time circulating RBCs undergo the *process of senescence* or aging. Each day, 1% of the old RBCs in circulation are taken out by a system of fixed macrophages in the body known as the **reticuloendothelial system (RES)** or the **mononuclear phagocytic system (MPS)**. Modifications of the RBC membrane proteins that normally occur during RBC senescence play a role in enhancing the recognition of aging red cells and their removal by phagocytic cells of the MPS. These RBCs are replaced by the daily release of 1% of the younger RBCs (reticulocytes) from the bone marrow storage pool. As erythrocytes become older, certain glycolytic enzymes decrease in activity, resulting in a decrease in the production of energy and loss of deformability. At a certain critical point, the RBCs are no longer able to traverse the microvasculature and are phagocytized by the RES cells. Although RES cells are located in various organs and throughout the body, those of the spleen, called **liver cells**, are the most sensitive detectors of RBC abnormalities.

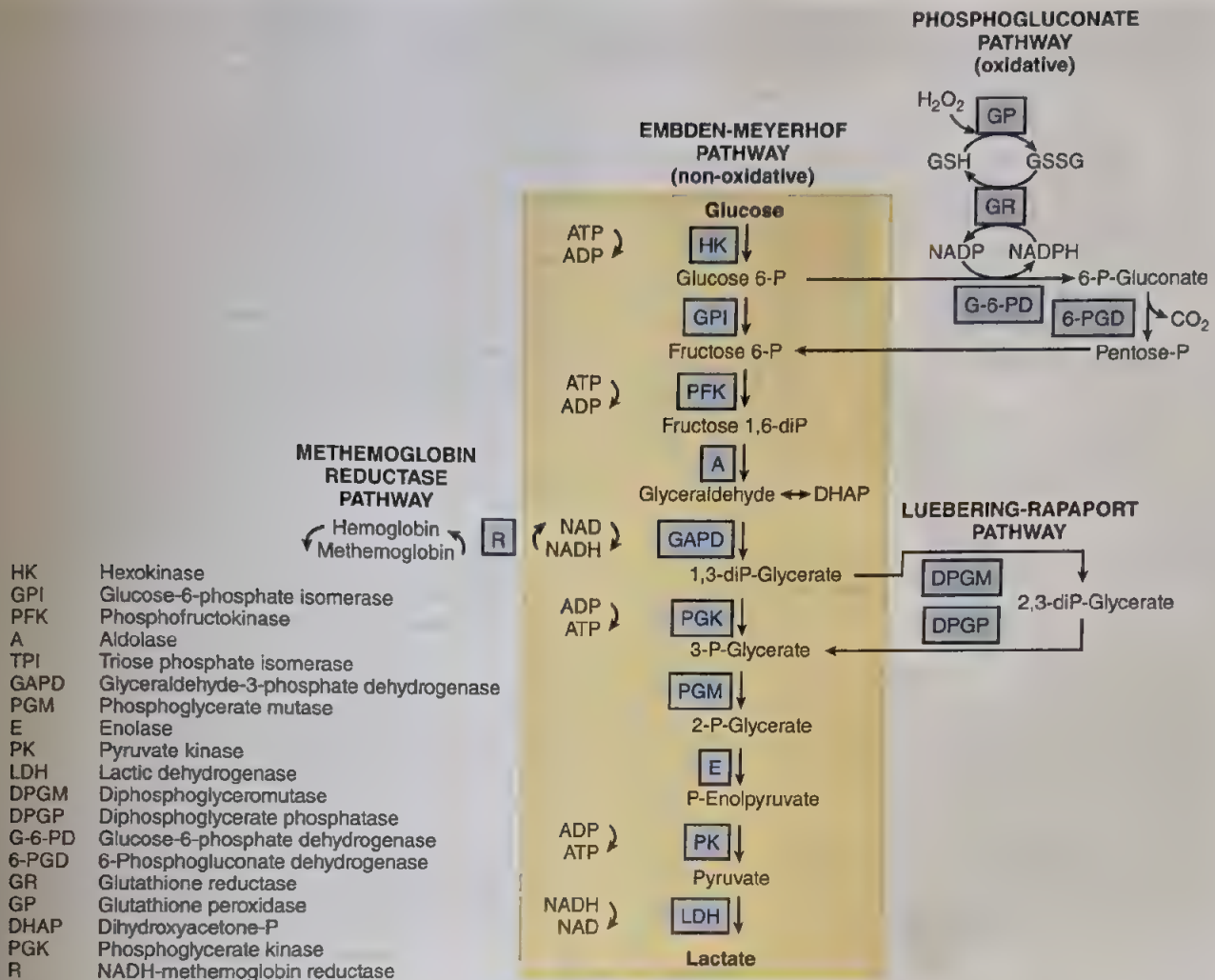


FIGURE 2-16 Red cell metabolism.



FIGURE 2-17 Scanning electron micrograph (SEM) of a normal red cell.

Phosphatidylserine is an aminophospholipid that is known to play a crucial role in mediating the recognition of senescent RBC, serving as a signal for phagocytosis. An excess of cholesterol inhibits the phosphatidylserine exposure; whereas cholesterol depletion increases it. This could lead to increased susceptibility to the clearance of the senescent RBCs.¹⁴

Extravascular Hemolysis

Ninety percent of the destruction of senescent RBCs occurs by the process of **extravascular hemolysis** (Fig. 2-18). During this process, old or damaged RBCs are phagocytized by the RES cells

and digested by their lysosomes. The hemoglobin molecules are disassembled and broken down into their various components. The iron recovered is salvaged and returned by the plasma protein carrier, transferrin, to the erythroid precursors in the marrow for synthesis of the new hemoglobin. Globin is broken down into amino acids and redirected to the amino acid pool of the body. Finally, the protoporphyrin ring of heme is disassembled, and its α carbon exhaled in the form of carbon monoxide. The opened tetrapyrrole, biliverdin, is converted to bilirubin and carried by the plasma protein albumin to the liver.²⁴ In the liver, bilirubin is conjugated to bilirubin glucuronide and excreted along with bile into the intestines. Here, it is converted further, through bacterial action, into urobilinogen (stercobilinogen) and excreted in the stool. A small amount of urobilinogen is reabsorbed through enterohepatic circulation, filtered by the kidneys, and excreted in small amounts in the urine. Both unconjugated (prehepatic) and conjugated (posthepatic) bilirubin can be measured in the plasma as indirect and direct bilirubin, respectively, and used to monitor the amount of hemolysis.²⁴

Intravascular Hemolysis

Only 5% to 10% of normal RBC destruction occurs through intravascular hemolysis (Fig. 2-19).²⁴ During this process,

SUMMARY CHART

- Glycophorin is the major integral membrane protein, representing 20% of the total RBC membrane protein.
- Spectrin is the most abundant peripheral protein of the RBC membrane cytoskeleton, comprising 25% to 30% of the total membrane protein and 75% of the peripheral protein.
- The loss of ATP and subsequent decrease in the phosphorylation of spectrin lead to a decrease in deformability and RBC survival.
- The RBC membrane is freely permeable to water and anions while relatively impermeable to cations (Na^+ , K^+).
- The erythrocyte intracellular-to-extracellular ratios for sodium and potassium are 1:12 and 25:1, respectively.
- RBC membrane lipids consist of a bilayer of phospholipids interspersed with molecules of unesterified cholesterol and glycolipids present in equimolar quantities.
- Cholesterol comprises 25% of the RBC membrane lipid and is present in a 1:1 molar ratio with phospholipids.
- Hemoglobin is a conjugated globular protein consisting of globin (two pairs of polypeptide chains) and four heme groups, each of which contains a protoporphyrin ring plus ferrous iron.
- Normal hemoglobin synthesis is dependent on adequate iron delivery and supply, adequate synthesis of protoporphyrins, and adequate globin synthesis.
- Iron is delivered to the membrane of the RBC precursor by the protein carrier transferrin; two-thirds of total body iron is bound to heme in the hemoglobin molecule.
- Globin chain synthesis occurs on RBC-specific cytoplasmic ribosomes, which are initiated from the gene inheritance.
- Normal adult hemoglobin consists of 95% to 97% HbA, 2% to 3% HbA₂, and 1% to 2% HbF.
- The primary function of hemoglobin is delivery and release of oxygen to the tissues and facilitation of carbon dioxide excretion.
- A shift to the left of the hemoglobin–oxygen dissociation curve results in a decrease in oxygen delivery to the tissues.
- A shift to the right of the hemoglobin–oxygen dissociation curve results in a decrease of oxygen affinity for hemoglobin and increased oxygen delivery to the tissues.
- Abnormal hemoglobins, which are unable to carry oxygen, include carboxyhemoglobin, methemoglobin, and sulfhemoglobin.
- Ninety percent of ATP needed for RBC survival is generated via the Embden–Meyerhof glycolytic pathway.
- Ninety percent of RBC senescence occurs by extravascular hemolysis, whereas 5% to 10% occurs through intravascular hemolysis.

CASE STUDY 2-1

A patient with anemia presents to their primary care physician. The patient's history is significant for a decreased RBC count for several years. The physician has been monitoring the patient closely with laboratory analysis during this time. To date, the patient has not required a blood transfusion.

QUESTIONS

1. With a decreased number of RBCs, what functionality is hindered within this patient?
2. How is it possible that this patient is handling their condition well without the need for a blood transfusion?
3. If tested, would 2,3-BPG levels be high or low in this patient?

ANSWERS

1. The patient is at highest risk for decreased oxygen levels without enough red blood cells present, and the hemoglobin within them, to carry oxygen to the tissues.
2. In chronic, milder cases of anemia, patient cells might demonstrate a shift to the right in the oxygen dissociation curve, enabling the fewer red blood cells present to deliver oxygen to the tissues more efficiently.
3. With a shift to the right in the oxygen dissociation curve, you would expect to see increased 2,3-BPG levels.

REVIEW QUESTIONS

- Which of the following is not a process affecting RBC survival and function?
 - Integrity of RBC cellular membrane
 - Cell metabolism
 - Intravascular hemolysis
 - Hemoglobin structure
- Which abnormal RBC is not caused by a structural membrane defect?
 - Spherocyte
 - Target cell
 - Siderocyte
 - Acanthocyte
- Which list represents the complete set of processes necessary for normal hemoglobin production?
 - Iron delivery and supply, synthesis of protoporphyrins, and globin synthesis
 - Iron salvage, synthesis of conjugated bilirubin, and haptoglobin synthesis
 - Iron accumulation, synthesis of hemopexin, and globin catabolism
 - Iron catabolism, synthesis of uroporphyrinogen, and ferritin synthesis
- What is the correct list for the number and type of globin chains in normal adult hemoglobin?
 - Four α , two β , and two δ chains
 - Two α and two non- α chains
 - Two α , four β , one δ , and one ϵ chain
 - Two α , two β , two δ , and one ϵ chain
- What is the composition of normal adult hemoglobin?
 - 92% to 95% HbA₁; 5% to 8% HbA₂; 1% to 2% HbF
 - 90% to 92% HbA₁; 2% to 3% HbA₂; 2% to 5% HbF
 - 80% to 85% HbA₁; 2% to 3% HbA₂; 1% to 2% HbF
 - 95% to 97% HbA₁; 2% to 3% HbA₂; 1% to 2% HbF
- Which of the following cells is caused by iron accumulation?
 - Acanthocyte
 - Ringed sideroblast
 - Burr cell
 - Bite cell
- Which of the following is a list of abnormal hemoglobins that are unable to transport or deliver oxygen?
 - Carboxyhemoglobin and methemoglobin
 - Methemoglobin and fetal hemoglobin
 - Carboxyhemoglobin, sulfhemoglobin, and fetal hemoglobin
 - Carboxyhemoglobin, methemoglobin, and sulfhemoglobin
- Which metabolic pathway generates 90% of the ATP needed by RBCs?
 - Methemoglobin reductase pathway
 - Hexose monophosphate shunt
 - Embden–Meyerhof pathway
 - Leubering–Rapaport shunt
- What steps occur in the extravascular breakdown of senescent RBCs?
 - RES cells phagocytize red cells; iron is coupled to transferrin and returned to marrow; globin is returned to amino acid pool; biliverdin is converted to bilirubin; bilirubin is coupled to albumin and transported to liver; bilirubin glucuronide is converted to urobilinogen and excreted.
 - RBCs break down in lumen of vessel; the haptoglobin–hemoglobin complex goes to the liver; unbound hemoglobin dimers are excreted through the kidney as hemosiderin, hemoglobin, or methemoglobin; haptoglobin is broken down to be excreted as urobilinogen
 - RES cells phagocytize red cells; iron is coupled to transferrin and returned to marrow; globin is returned to amino acid pool; the haptoglobin–hemoglobin complex goes to liver; unbound hemoglobin dimers are excreted through kidney as hemosiderin, hemoglobin, or methemoglobin; haptoglobin is broken down to be excreted as urobilinogen.
 - RBCs break down in lumen of vessel; haptoglobin picks up dissociated hemoglobin; the haptoglobin–hemoglobin complex goes to the liver; biliverdin is converted to bilirubin; bilirubin is coupled to albumin and transported to liver; bilirubin glucuronide is converted to urobilinogen and excreted.

REVIEW QUESTIONS—cont'd

10. What steps occur in the intravascular breakdown of senescent RBCs?
 - a. RES cells phagocytize red cells; iron is coupled to transferrin and returned to marrow; globin is returned to amino acid pool; biliverdin is converted to bilirubin; bilirubin is coupled to albumin and transported to liver; bilirubin glucuronide is converted to urobilinogen and excreted.
 - b. RBCs break down in lumen of vessel; the haptoglobin-hemoglobin complex goes to the liver; unbound hemoglobin dimers are excreted through the kidney as hemosiderin, hemoglobin, or methemoglobin; haptoglobin is broken down to be excreted as urobilinogen.
 - c. RES cells phagocytize red cells; iron is coupled to transferrin and returned to marrow; globin is returned to amino acid pool; the haptoglobin-hemoglobin complex goes to liver; unbound hemoglobin dimers are excreted through kidney as hemosiderin, hemoglobin, or methemoglobin; haptoglobin is broken down to be excreted as urobilinogen.
 - d. RBCs break down in lumen of vessel; haptoglobin picks up dissociated hemoglobin; the haptoglobin-hemoglobin complex goes to the liver; biliverdin is converted to bilirubin; bilirubin is coupled to albumin and transported to liver; bilirubin glucuronide is converted to urobilinogen and excreted.
11. The membrane protein spectrin is important for which of the following?
 - a. Maintaining the RBC's net negative charge
 - b. Maintaining the RBC's membrane integrity
 - c. Maintaining the RBC's regulated volume
 - d. Maintaining the RBC's net positive charge
12. If the RBC cannot deform, what is a consequence?
 - a. The RBC will undergo intravascular hemolysis.
 - b. The RBC will lose too much cellular volume.
 - c. The RBC may not make it through the RES vasculature.
 - d. The RBC will not be able to generate the required energy needed for functionality.
13. The primary function of hemoglobin is to:
 - a. Generate ATP for the RBC
 - b. Fuel the cation pump for the RBC
 - c. Maintain RBC membrane deformability
 - d. Carry and deliver oxygen
14. When there is a shift to the right in the oxygen dissociation curve, which of the following is true?
 - a. Hemoglobin's affinity for delivering oxygen is increased.
 - b. Hemoglobin's affinity for holding on to oxygen is increased.
 - c. 2-3,DPG levels are decreased.
 - d. The patient may be hypothermic.

See answers at the back of this book.

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Bone Marrow Structure and Function

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CHAPTER OUTLINE

Bone Marrow Structure

Erythropoiesis
Granulopoiesis
Megakaryopoiesis
Lymphopoiesis
Stem Cells
Hematogones
Marrow Stromal Cells
Mast Cells
Bone-Forming Cells

Bone Marrow Function

Indications for Bone Marrow Studies

Obtaining and Preparing Bone Marrow for Hematologic Studies

Equipment
Aspiration
Preparation of Bone Marrow Aspirate
Histologic Marrow Particle Preparation
Bone Marrow Core Biopsy
Preparation of Trephine Biopsy

Bone Marrow Examination

Estimation of Bone Marrow Cellularity
Bone Marrow Differential Count

Bone Marrow and Peripheral Blood Interpretation Based on Cellularity and M:E Ratio Changes
Bone Marrow Iron Stores

Bone Marrow Report

Summary Chart

Case Study 3-1

Case Study 3-2

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 3-1 Define hematopoiesis.
- 3-2 Contrast the proliferating pool with the maturation storage pool in granulopoiesis.
- 3-3 Explain the characteristics that make marrow stem cells unique.
- 3-4 Describe antigen-dependent and antigen-independent proliferation.
- 3-5 Differentiate red marrow from yellow marrow.
- 3-6 Explain the main function of the bone marrow.
- 3-7 List the conditions that warrant bone marrow studies.
- 3-8 List the anatomical sites for bone marrow collection.
- 3-9 Detail the medical laboratory scientist's role in the bone marrow aspiration procedure.
- 3-10 Describe the normal cellular composition of adult bone marrow.
- 3-11 State the difference between bone marrow aspirate and biopsy.
- 3-12 Explain how iron is stored in the bone marrow and the stains used to identify iron on a bone marrow smear.

The hematopoietic system consists of the bone marrow, liver, spleen, lymph nodes, and thymus. Blood cell production and maturation, or **hematopoiesis**, can be seen at different anatomic locations (e.g., yolk sac, liver, spleen, axial and radial bones), depending on the gestational and postnatal period. In healthy adults, hematopoiesis is seen mainly in the bone marrow. The bone marrow-derived pluripotent hematopoietic stem cells, under the influence of various cytokines, growth factors, or both, differentiate into **myeloid** (granulocytes, monocytes, megakaryocytes, erythrocytes) and **lymphoid** cell lineages. Benign conditions and malignant diseases related to these cells are termed **hematolymphoid disorders**. Nonhematolymphoid diseases can also involve the bone marrow; therefore, examination of the bone marrow has a wide application in clinical medicine.

Because hematologic diseases involving the bone marrow can cause morphological abnormalities of the peripheral

blood cells, the bone marrow examination should be interpreted in conjunction with a peripheral smear examination. Bone marrow aspiration and biopsy are usually indicated for the definitive diagnosis of many hematologic disorders. Contraindications include severe **hemophilia** and severe **disseminated intravascular coagulopathy (DIC)**.¹ **Thrombocytopenia** is generally not a contraindication to bone marrow aspiration and biopsy because platelet transfusion can be performed, if clinically warranted, before the procedure.¹

Bone Marrow Structure

The bone marrow is one of the body's largest organs, representing 3.4% to 6% of total body weight and averaging about 1,500 grams in adults.¹ The hematopoietic marrow is organized around the bone vasculature.² An artery entering the bone branches out toward the periphery to specialized

vascular spaces called **sinuses** (Figure 3-1). Several sinuses combine in a collecting sinus, forming a central vein that returns into the systemic circulation. Hematopoietic cords, in which hematopoiesis takes place, lie just outside of the sinuses. After maturation in the cords, the hematopoietic cells cross the walls of the sinuses and enter the blood.³ Hematopoietic cell colonies are compartmentalized in the cords. Structurally, bone marrow consists of hematopoietic cells (erythroid, myeloid, lymphoid, and megakaryocyte), adipose tissue, bone and its cells (osteoblasts and osteoclasts), and stroma.³

Erythropoiesis

Erythropoiesis is the process of red blood cell production. This process takes place in distinct anatomic units called erythropoietic islands⁴ (Figure 3-2). Each island consists of a macrophage surrounded by a cluster of maturing erythroblasts.⁵ Hemoglobin synthesis occurs as early as the pronormoblastic stage, but most hemoglobin synthesis occurs in the polychromatophilic stage. The average life span of circulating red blood cells is 120 days.

Granulopoiesis

Granulopoiesis, or the differentiation and maturation of granulocytes, takes place in the hematopoietic cords of the bone marrow. In the bone marrow, the committed granulocytic progenitor cells that come from the hematopoietic stem cells will undergo proliferation and differentiation into neutrophils.⁶ Early granulocytic precursors are located deep in the cords and around the bone trabeculae. In Figure 3-3, granulopoiesis is less conspicuously oriented toward a distinct reticulum cell yet may be recognized as an entity. Neutrophils in the marrow can be divided into the **proliferating pool** and the

maturation storage pool. The proliferating pool includes myeloblasts, promyelocytes, and myelocytes.^{5,6} These cells which spend 3 to 6 days in this pool, are capable of DNA synthesis and undergo cell division. The maturation storage pool consists of nondividing metamyelocytes, bands, and segmented neutrophils.^{5,6} The cells typically spend 5 to 7 days in this pool before entering into the circulation.⁵ Depending on the demand, the cells from the storage pool (representing 15 to 20 times as many cells as in the blood) can be released into the peripheral blood, thus increasing the total white blood cell (WBC) count in minutes or hours.⁵ The average life span of circulating neutrophils is 6 to 10 hours.

Megakaryopoiesis

Megakaryopoiesis, the production of megakaryocytes, or platelets, occurs adjacent to the sinus endothelium.³ The megakaryocytes protrude as small cytoplasmic processes through the vascular wall, delivering platelets directly into the sinusoidal blood.⁷ Megakaryocytes are situated close to marrow sinuses and, through a mechanism that is not entirely understood, shed platelets into the circulation (Figure 3-4). Approximately 5 days are required for the megakaryocytes to produce platelets.⁷ Two-thirds of shed platelets are present in the circulation, whereas one-third are sequestered in the spleen. The average life span of platelets in the circulation is approximately 10 days.

Lymphopoiesis

Lymphocytes and plasma cells are minor components of nucleated cells in normal bone marrow. Lymphocyte production, or **lymphopoiesis**, is compartmentalized in lymphoid follicles, and lymphocytes are randomly dispersed throughout the cords³

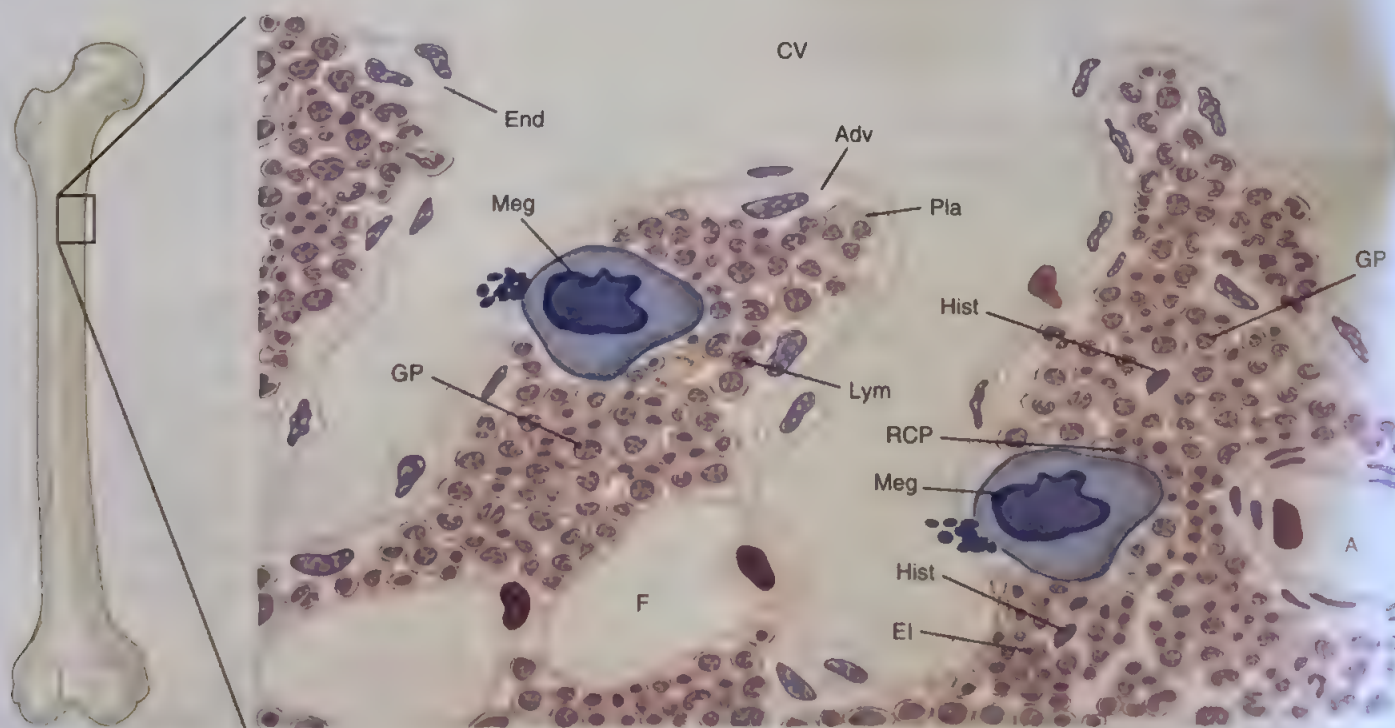


FIGURE 3-1 Graphic presentation of hematopoietic tissue. The vascular compartment consists of arteriole (A) and central sinus (CV). The venous sinusoids are lined by endothelial cells (End), and their wall outside is supported by adventitial reticulum cells (Adv). Fat tissue (F) is part of the marrow. The compartmentalization of the hematopoiesis is represented by areas of granulopoiesis (GP), areas of erythropoiesis (RCP), and erythropoietic islands (EI) with their nutrient histiocyte (Hist). The megakaryocytes protrude with small cytoplasmic projections through the vascular wall (Meg). Lymphocytes (Lym) are randomly scattered among the hematopoietic cells, whereas plasma cells (Pla) are usually situated along the vascular wall.

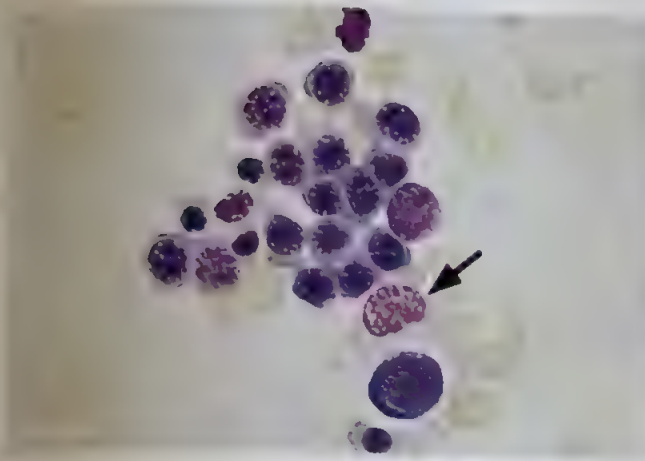


FIGURE 3-2 Erythropoietic island composed mainly of polychromatophilic normoblasts. The nutrient-histiocyte (arrow) is slightly displaced off its central position by smearing of the particle. Its cytoplasmic slender processes envelop a basophilic normoblast, establishing intimate contact with the maturing red cell precursor. (Wright-Giemsa, magnification $\times 600$)

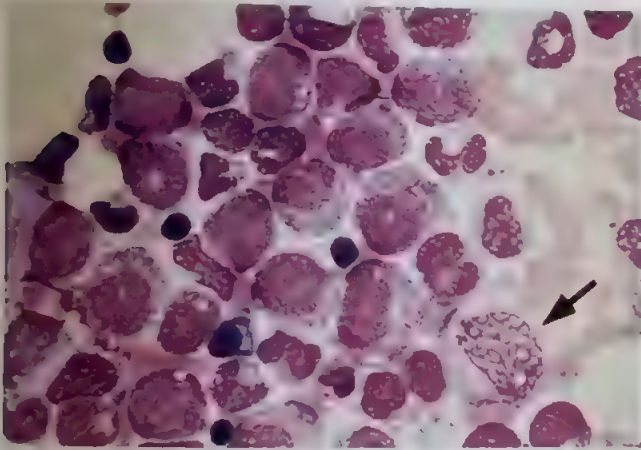


FIGURE 3-3 Compartment of granulopoiesis. A reticulum cell (arrow) with open reticulated chromatin and light blue cytoplasm containing dustlike fine granules is situated among numerous granulocytic precursors, especially myelocytes. (Wright-Giemsa, magnification $\times 600$)

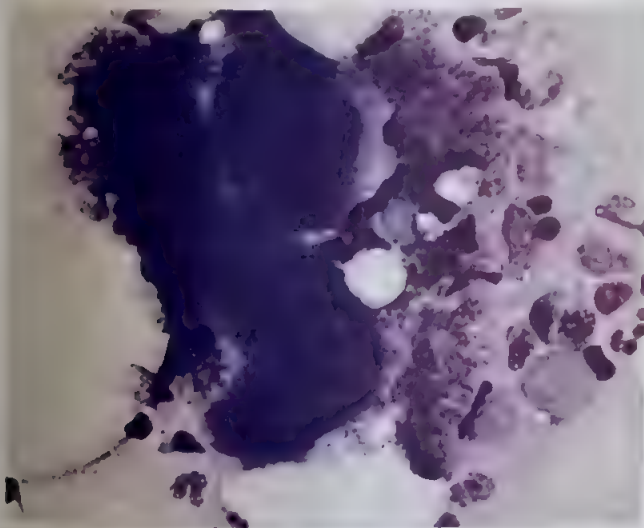


FIGURE 3-4 Mature megakaryocytes releasing proplatelets (packages of platelets). (Wright-Giemsa, magnification $\times 600$)

The lymphoid follicles (most often seen in elderly individuals) are unevenly distributed and tend to influence the variability of the lymphocyte count in aspirated bone marrow samples⁹ (Figure 3-5). Plasma cells are situated along the vascular wall.

Stem Cells

The marrow stem cells have two unique biological characteristics: self-renewal and multilineage differentiation. These stem cells can be further subcategorized as **pluripotential stem cells** (which give rise to many different cell lines) and **committed stem cells**. Because committed stem cells are dedicated by lineage to one cell type and do not have the potential of self-renewal, they are also called **progenitor cells**. A progenitor cell is a cell committed to a single line of proliferation and differentiation. The marrow stem cells on Wright's-stained smears are morphologically indistinguishable from small lymphocytes. In the presence of appropriate growth factors, the stem cells differentiate into myeloid and lymphoid cells that carry tissue and humoral immune functions, respectively. Some lymphoid progenitor cells produced in the marrow mature in the thymus as T lymphocytes; others are produced and continue their maturation and differentiation in bone marrow as B lymphocytes from the 12th gestational week throughout life.¹⁰ Therefore, the bone marrow and thymus are primary lymphoid organs of *antigen-independent* progenitor lymphoid cell proliferation and differentiation, which gives rise to new lymphocytes. These new lymphocytes may then populate the secondary lymphoid organs such as lymph nodes, spleen, and lymphoid apparatus of the gastrointestinal tract. Under appropriate stimulation, the mature lymphocytes of the peripheral lymphoid organs undergo *antigen-dependent* effector cell proliferation, resulting in cytokines and antibody production from T and B lymphocytes, respectively.¹¹

Hematogones

Hematogones are normal cellular constituents of bone marrow that resemble small- to intermediate-sized lymphocytes.¹² They range in size from 10 to 20 μm and have a high nuclear-to-cytoplasmic ratio and smooth, smudged

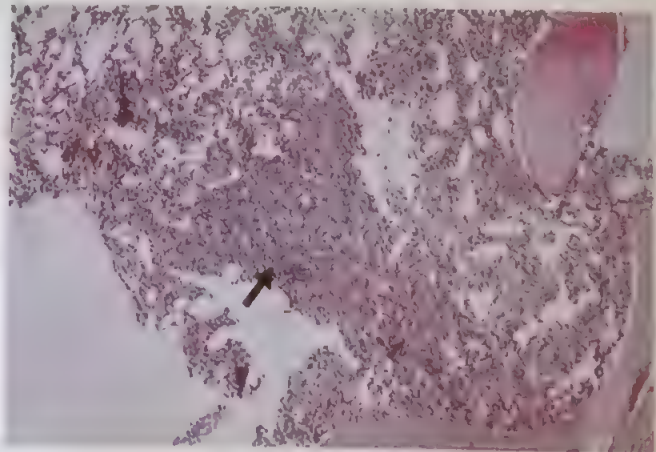


FIGURE 3-5 A lymphocytic nodule (follicle) in bone marrow as shown here may alter very significantly the marrow differential count when aspirated and give a false impression of lymphocytic malignancy. (H&E, magnification $\times 200$)

homogeneous chromatin.¹² Their cytoplasm is deeply basophilic and devoid of any granules or vacuoles (Figure 3-6). Hematogones are thought to be committed progenitor cells of lymphoid lineage, B-lymphocyte precursors.^{12,13,14} Their numbers are increased in normal infants, older children, and sometimes in adults (in regenerative marrow after chemotherapy and bone marrow transplantation), as well as in marrows of children with neuroblastoma with or without metastasis, iron-deficiency anemia, and idiopathic thrombocytopenic purpura.^{14,15} These cells closely resemble blasts of lymphoblastic leukemia, and laboratory personnel should be aware of the conditions in which their numbers may be increased.¹³

Marrow Stromal Cells

The meshwork of **stromal cells** in which the hematopoietic cells are suspended is in a delicate semifluid state and is composed of reticulum cells, histiocytes, fat cells, and endothelial cells.¹⁶ The reticulum cells are associated with fibers that can be visualized after silver staining. They are adjacent to the sinus endothelial cells, forming the outer part of the wall as an adventitial reticulum cell.¹⁶ Their fine cytoplasmic projections extend deep into the cords, contacting with similar projections of other cells.¹⁶ Occasionally, the nuclear region of these cells can be seen deep in the cords surrounded by granulopoiesis. Cytochemically, these cells are alkaline-phosphatase positive (Figure 3-7). Histiocytes or macrophages are seen as perisinusoidal cells related to the bone marrow-blood barrier.¹⁷ The macrophages are the central storage area of the erythropoietic islands in the bone marrow. As storage nutrient cells that deliver iron to the growing immature erythroblasts, the storage macrophages send out long, slender cytoplasmic processes that envelop the erythroid precursors. This extensive and intimate contact with the maturing erythropoietic cells is necessary in transferring iron from the macrophage to the red cell precursors. As phagocytic cells, the macrophages also undergo hyperplasia when there is increased destruction of hematopoietic cells. Histochemically, the macrophages are acid-phosphatase positive (Figure 3-8).

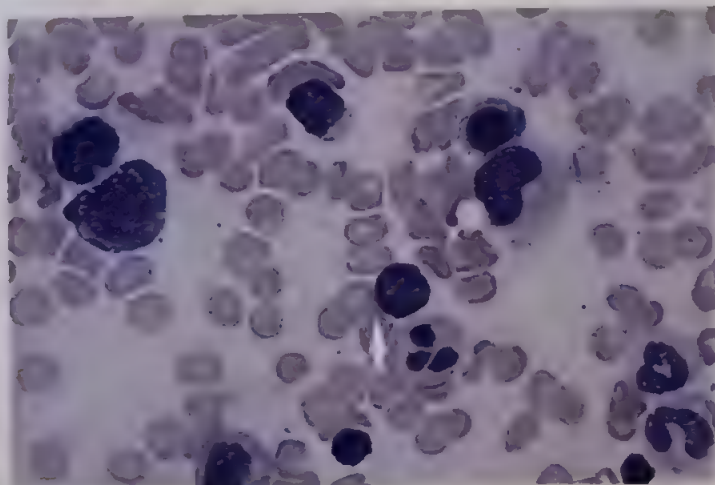


FIGURE 3-6 An arrow points toward a hematogone showing high nuclear-to-cytoplasmic ratio, homogeneous chromatin, and scant cytoplasm. It can be confused with a blast of lymphoblastic leukemia. (Wright-Giemsa, magnification $\times 1,000$)

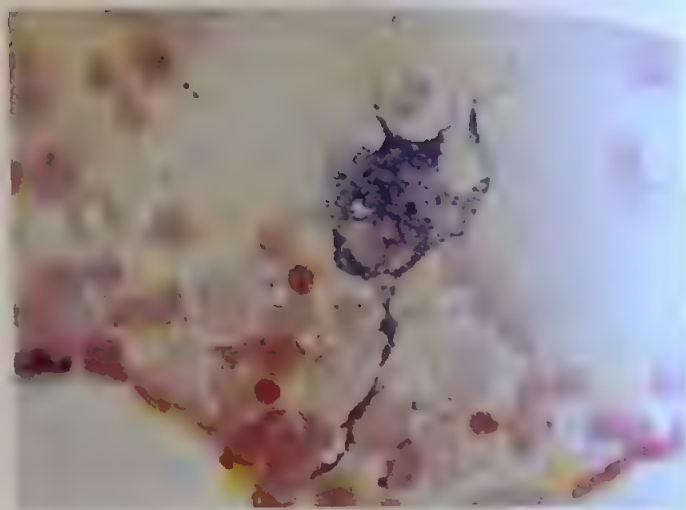


FIGURE 3-7 An alkaline phosphatase-stained reticulum cell extends its slender cytoplasmic projections deep in the hemopoietic cord, maintaining an intimate contact with granulopoiesis. The background cells are stained with neutral red. (magnification $\times 600$)

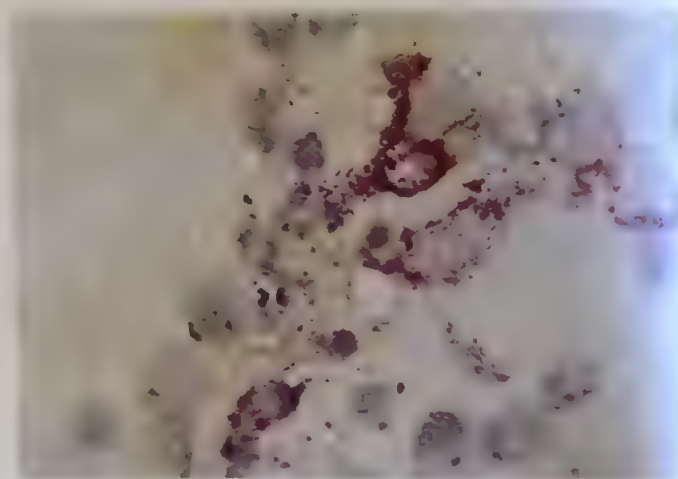


FIGURE 3-8 Two acid phosphatase-positive macrophages in bone marrow of a patient treated with chemotherapeutic agents. Macrophages are also scavengers and cleaners of the hematopoietic tissue, so they increase in number during massive destruction of hematopoietic cells. (magnification $\times 600$)

The stromal cells produce an extracellular matrix composed of collagens, glycoproteins, proteoglycans, and other proteins.¹⁷ This extracellular matrix is essential in maintaining normal renewal and differentiation of marrow cells.¹⁷ The bone marrow consists of **red marrow** (hematopoietic active) and **fatty yellow marrow** (hematopoietic inactive). The marrow cellularity is estimated as a percentage of red marrow to total marrow. The fatty yellow marrow (adipose cells) can vary in amount according to the age of the patient and the skeletal location from where the marrow is obtained. In young children, most of the marrow is composed of red (hematopoietic active) marrow with only a few fat cells present. The adipose tissue gradually increases after 4 years of age. In adults, fat cells average about 50% of the total marrow volume in the vertebrae and flat bones of the pelvis.¹⁸ The marrow and the extracellular matrix are dynamic tissues similar to the hematopoietic tissue, and these may be altered rapidly in disease states.

The bone marrow is a highly vascularized tissue from which endothelial cells can occasionally be aspirated. Endothelial cells are more visible in hypoplastic marrows and should not be mistaken for metastatic tumors (Figure 3-9).

Mast Cells

Tissue mast cells (Figure 3-10) measure 6 to 12 μm in diameter, are connective tissue cells of mesenchymal origin, and are normally present in the bone marrow in varying numbers.¹⁹ They have a round or oval reticular nucleus and abundant blue-purple granules that obscure the nucleus. Their granules, in addition to all other substances that are present in the granules of basophils, contain serotonin and proteolytic enzymes.²⁰ The numbers of mast cells can be increased in chronic infections, autoimmune diseases, chronic lymphoproliferative disorders, and especially in systemic mastocytosis.^{19, 20}

Bone-Forming Cells

In marrow aspirates, cells are occasionally seen originating from bone tissue. **Osteoblasts** are bone matrix-synthesizing cells usually found in groups.²¹ They are up to 30 μm in diameter

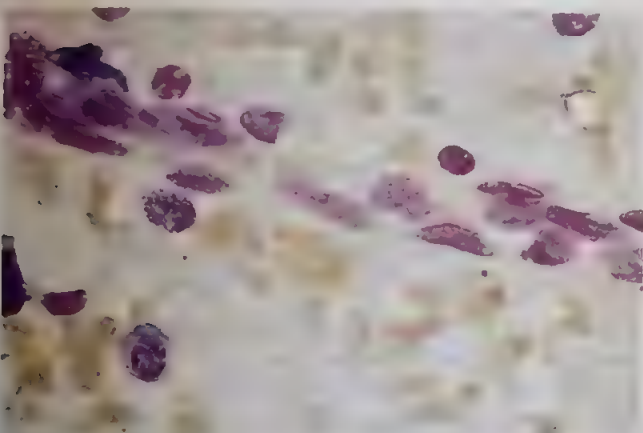


FIGURE 3-9 A string of endothelial cells aspirated from hypocellular marrow. The nuclei are elongated and slightly tapered. The cytoplasm is transparent and barely visible. (Wright-Giemsa, magnification $\times 600$)

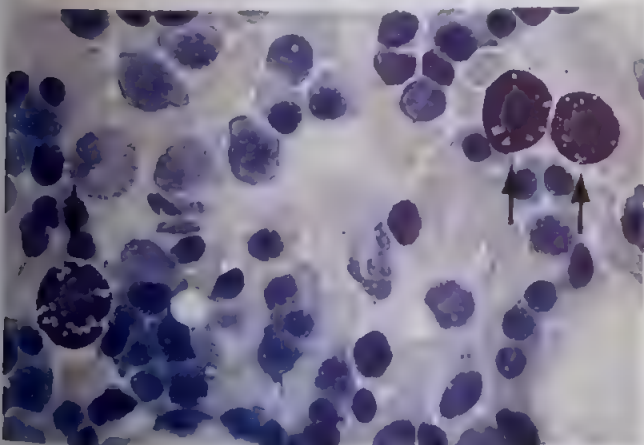


FIGURE 3-10 Three mast cells, known also as tissue basophils, are shown in this marrow aspirate in a background of erythroid hyperplasia. Numerous regular round granules fill their cytoplasm and obscure the nuclear details. (Wright-Giemsa, magnification $\times 600$)

and resemble plasma cells.²¹ The osteoblast nucleus has a fine chromatin pattern with a prominent nucleolus. A perinuclear halo, detached from the nuclear membrane with a cytoplasmic bridge, represents the Golgi apparatus area. Osteoblasts are alkaline-phosphatase positive. They are characteristically seen in bone marrow aspirates of children and patients with metabolic bone diseases.¹⁸

Osteoclasts, or bone remodeling cells, are multinucleated giant cells more than 100 μm in diameter, resembling megakaryocytes.²² The nuclei of the osteoclasts are separate from each other and may have nucleoli (compared with the megakaryocyte nucleus, which is multilobed).^{22, 23} Their cytoplasm is well delineated and finely granular.

Bone Marrow Function

The main function of the marrow is to supply mature hematopoietic cells into the peripheral blood in a steady-state condition as well as to respond to increased demands. A semidormant pool of pluripotential stem cells maintains a self-renewal property. Granulocytic, monocytic, eosinophilic, erythroid, and megakaryocyte progenitor cells are influenced in their differentiation by **colony-stimulating factors (CSFs)**.^{24, 25} CSFs are produced by T lymphocytes—as well as stromal cells, fibroblasts, endothelial cells, and macrophages—when stimulated by monocyte interleukin-1 (IL-1) and tumor necrosis factor (TNF). Some CSFs, such as IL-3 and granulocyte-monocyte CSF, have a broad influence and are required throughout proliferation and differentiation of progenitor cells. Others, which include granulocyte, monocyte, and eosinophil CSFs, are lineage-specific and regulate division and differentiation only of corresponding, committed progenitor cells.

Erythropoiesis is influenced by EPO produced in the kidney.²⁴ In the process of cell egression from the cords to the circulation, several releasing factors are identified. The best characterized of these are **granulocyte colony-stimulating factor (G-CSF)** and **granulocyte-macrophage colony-stimulating factor (GM-CSF)**, but other factors may play a role.^{24, 25} The endothelial lining of the sinusoids forms a continuous, veil-like wall through which the mature cells migrate from extravascular sites into the circulation.³ This is accomplished by close contact between mature hematopoietic cells and endothelial cells. A transient migration pore is formed during such contact through which the mature cells pass into the circulation without loss of plasma to the extravascular pool.²⁶ It is evident that the bone marrow is subjected to a complex regulation by many cellular and humoral systems of the body, and any disease that affects these systems is likely to affect hematopoiesis.²⁶

Indications for Bone Marrow Studies

In 1929, Arinkin introduced bone marrow studies in the diagnosis of hematopoietic disorders.²⁷ Once a formidable task, obtaining bone marrow tissue has become, with current improved techniques, a standard procedure. Several techniques have been devised, each having its own merits

and limitations.²⁸ Bone marrow aspiration and bone marrow biopsy are usually performed concurrently.¹

Although obtaining bone marrow for examination carries little procedural risk for the patient, the procedure is costly and can be quite painful. For this reason, bone marrow studies should be performed only when clearly indicated or whenever the physician expects a beneficial diagnostic result for their patient (Table 3-1). Hematologic diseases primarily affecting the bone marrow and causing a decrease or increase of any cellular blood elements are among the most common indications. It is not unusual for more than one blood element to be increased or decreased, as occurs in leukemias and some refractory anemias. In these situations, a bone marrow study offers specific information, and it is usually required for a complete hematologic workup, diagnosis, and treatment planning.²⁸

Systemic diseases may affect the bone marrow secondarily and require bone marrow studies for diagnosis or monitoring patients' conditions. Patients having any of the solid malignant tumors may undergo bone marrow studies when the initial diagnosis is established for evaluation of the degree of tumor spread and staging of the disease. On occasion, a bone marrow study may result in a diagnosis of unsuspected metastatic malignant tumor. During the course of the malignant disease, additional marrow studies may be performed periodically to monitor the status of tumor burden and its therapeutic response.²⁸

Infections manifesting clinically as "fever of unknown origin" may exhibit granulomas, focal necrosis, or histiocytic proliferations. Intracytoplasmic organisms may be seen in the marrow. Material for morphological studies and bacterial cultures may be collected simultaneously during a single procedure. The suspected diagnoses of disseminated tuberculosis, fungal infections (particularly histoplasmosis and cryptococcosis), and some protozoan infections are frequently

confirmed through such studies. Hereditary and acquired conditions occasionally involve the bone marrow histiocytes (e.g., Gaucher's disease as shown in Figure 3-11, sea blue histiocytosis, hemophagocytic syndrome, and others). A simple procedure such as bone marrow aspiration or biopsy may establish the diagnosis.

ADVANCED CONTENT

Since bone marrow aspiration is an invasive procedure, pathologists are required to evaluate the medical necessity of the procedure by considering the patient's history, clinical differential diagnosis, and/or review of the peripheral blood morphological slides. At the time the bone marrow samples are obtained, review of cellular morphology must be done in a timely manner so samples can be examined appropriately.

CRITICAL THINKING QUESTION

3-1 Would an increased neutrophil concentration alone indicate the need for a bone marrow study?

See answers to all Critical Thinking Questions at the back of this book.

Obtaining and Preparing Bone Marrow for Hematologic Studies

The sites for bone marrow studies in adults are most commonly the posterior superior iliac crest, occasionally the sternum, and very rarely the anterior superior iliac crest and spinal processes or vertebral bodies²⁸ (Figure 3-12). Sternum aspiration should be avoided in children, as well as in patients with multiple myeloma and metastatic carcinoma, because these diseases can cause thinning and erosion of bone, thus

TABLE 3-1 Indications and Contraindications for a Bone Marrow Study

Indications	Contraindications
Hematologic <ul style="list-style-type: none"> Anemias, polycythemia Unexplained leukopenia or leukocytosis Presence of blasts, immature, or abnormal cells in the circulation Unexplained thrombocytopenia or thrombocytosis Evaluation of plasma cell disorders Evaluation of unexplained cytopenias 	Severe Bleeding Diatheses <ul style="list-style-type: none"> Severe hemophilia Severe disseminated intravascular coagulopathy
Systemic Diseases <ul style="list-style-type: none"> Staging and management of solid malignant tumors arising elsewhere in the body, such as lymphomas, carcinoma, and sarcomas Infections or fever of unknown origin, granulomas Hereditary or acquired metabolic disorders Systemic mast cell disease 	

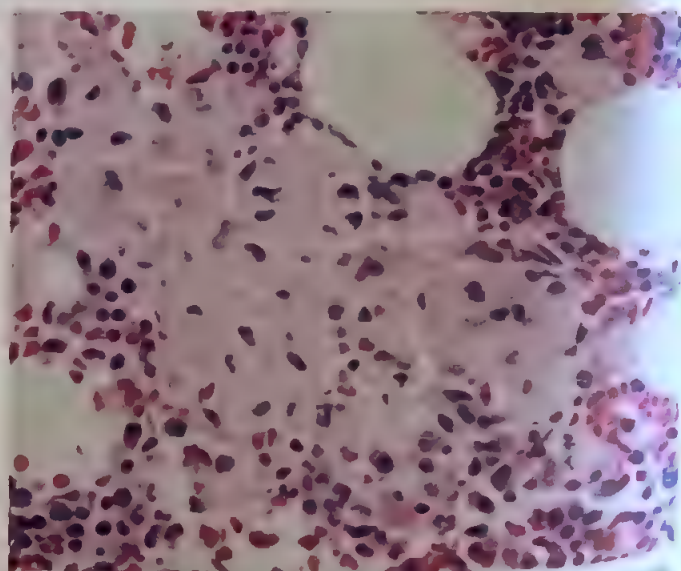


FIGURE 3-11 Bone marrow biopsy showing a collection of Gaucher cells (H&E, magnification $\times 600$)

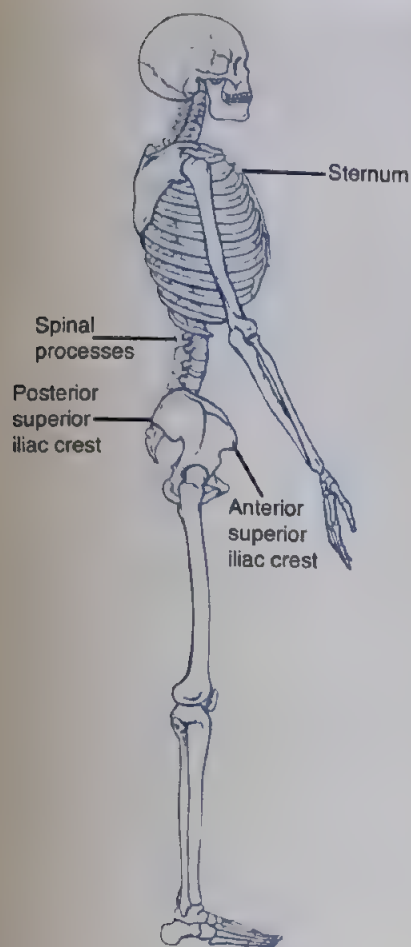


FIGURE 3-12 Common sites from which bone marrow is obtained for studies.

increasing the chance of perforation and causing potentially fatal cardiac complications.²⁸ Occasionally, when a localized bone lesion is visualized on x-ray or computed tomographic (CT) scan, a directed or "open" bone marrow biopsy of the lesion may be performed by a radiologist or surgeon in an operating room with the patient under anesthesia. In newborns and infants, a bone marrow sample can be obtained from the upper end of the tibial bone.

Before performing the procedure, the provider should inform the adult patient or the parent or guardian of a child of the procedure, its risks, and the expected benefits for the diagnostic process. The bone marrow procedure cannot be performed until a consent form is signed and witnessed by a second person, commonly the patient's nurse. The actual procedure is often performed with the assistance of a medical laboratory scientist. Although the provider performs the procedure and the nurse attends to the patient, the medical laboratory scientist gives full attention to the processing of the specimens to ensure that the samples are adequate. If they are not, the provider is informed immediately so that the procedure can be repeated before the patient is discharged. Samples are preserved appropriately for histologic, flow cytometric, cytogenetic, FISH (fluorescent in-situ hybridization) microbiologic, electron microscopic, molecular, and other studies as indicated in a particular case.^{1,28}

In experienced hands, complications of bone marrow biopsy and aspirate are very rare (0.1%). These rare complications include pain, bleeding or infection at the biopsy site, transient neuropathy, and osteomyelitis. After the procedure, the patient is advised to lie on the biopsy site, which should be reevaluated in 15 to 30 minutes for any bleeding or oozing.

Equipment

The instrument tray used to perform a bone marrow procedure should contain enough equipment to complete the procedure and to prepare the tissues obtained for the appropriate studies (Box 3-1). Complete bone marrow trays are sold as disposable equipment, which is convenient and avoids the risk of transmitting infectious diseases.

Several different styles of aspiration and trephine bone biopsy needles or instruments are commonly used. Most instruments used today are patterned on the needle introduced by Jamshidi.²⁸ These instruments are produced in several sizes for both adult and pediatric patients. An example of the Jamshidi bone marrow biopsy/aspiration needle for adults is shown in Figure 3-13. Modifications of the original aspiration and trephine needles have been developed by different companies and are manufactured as disposable equipment.

BOX 3-1 Supplies for Bone Marrow Aspiration and Biopsy

Required Materials

- 30-mL syringes
- 20-mL syringes
- 10-mL syringes
- 5-mL syringes
- 2% Lidocaine
- Prepodyne prep
- Alcohol (70%) or prep
- 23-Gauge needles
- 21-Gauge needles
- Bone marrow biopsy/aspiration needle 11-gauge × 4 in.
- Filter papers
- Buffered formalin 10% with a pH of about 6.8 or other fixative for histologic processing of bone biopsy and marrow particles
- Tube containing liquid EDTA anticoagulant
- One box of slides
- One slide folder
- One rubber bulb
- Pasteur pipet
- Petri dish
- Sterile blades
- Gloves (several pairs of different sizes)
- Sterile gauze and cotton balls
- Applicator sticks
- Bandage
- Culture bottles for bacterial culture. (Note: Save some bone marrow specimen in syringe for tuberculosis and fungal cultures, when indicated.)
- Pencil to label slides
- No. 11 Bard Parker blades

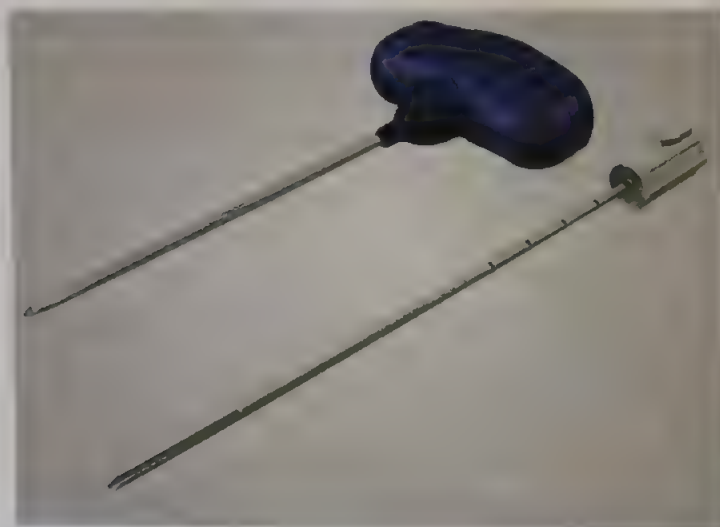


FIGURE 3-13 "Jamshidi Evolve™ Bone Marrow Biopsy Needle with Specimen Cradle." (Courtesy and © Becton, Dickinson and Company.)

Aspiration

A bone marrow aspiration may be performed as an independent procedure or in conjunction with a bone marrow biopsy.²⁹ The procedure can be performed in the outpatient setting in clinics or in the physician's office. As a rule, children and very apprehensive patients receive a mild sedative before the procedure.

The site selected is shaved, if needed, and washed with soap. Then an antiseptic is applied, and the area is draped with sterile towels. A local anesthetic such as 1% to 2% lidocaine (Xylocaine) is infiltrated into the skin, in the intervening tissues between the skin and bone, and in the periosteum of the bone from which the marrow is to be obtained. A cut of about 3 mm is made through the skin with a Bard-Parker blade to facilitate piercing skin and subcutaneous tissue.

The provider penetrates the bone cavity with an aspiration needle, assembled with guard and stylet locked in place.

When the marrow cavity is penetrated, the stylet is removed, a syringe is attached to the free end of the needle, and the plunger is quickly pulled, drawing 1.0 to 1.5 mL of marrow particles and sinusoidal blood into the syringe. Because the vacuum created in the syringe is important for rapid and efficient suctioning of the cells and particles, the syringe should be 10 mL or larger with a well-fitting plunger. Despite the use of local anesthesia, the patient normally experiences discomfort during the aspiration process (aspiration pain). Accomplishing the aspiration with a quick and continuous pull on the plunger diminishes the patient's discomfort and decreases the chance of clotting the specimen. A clotted specimen is useless for smear preparation because the fibrin threads strip the cytoplasm off the cells and hamper their spreading.

Keeping the volume of the initial aspirate small also prevents dilution of the sample with large amounts of sinusoidal blood, thus improving the quality of the aspirate. This first-aspirated material is used immediately for preparing smears. Additional aspirate may be obtained in separate syringes if needed for flow cytometry, chromosome studies, bacterial cultures, and other tests (Figure 3-14). Once an adequate aspirate is obtained, the quality of the smear depends entirely on the medical laboratory scientist's skill and speed in preparing the smears and preserving the morphology of the marrow cells. Part of the first aspirate is used for the preparation of direct and marrow particle smears. Another portion is placed in an ethylene diaminetetraacetic acid (EDTA) anticoagulant-containing tube for use as a particle preparation. If some aspirate remains, it can be left to clot. The clot may be fixed in 10% buffered formalin or another chosen fixative and processed for histologic examination. The preferred anticoagulants for ancillary studies performed on the bone marrow aspirate are EDTA for flow cytometry and molecular, and sodium heparin for cytogenetics and FISH. If the aspiration attempt is unsuccessful (a "dry tap"), an additional core biopsy

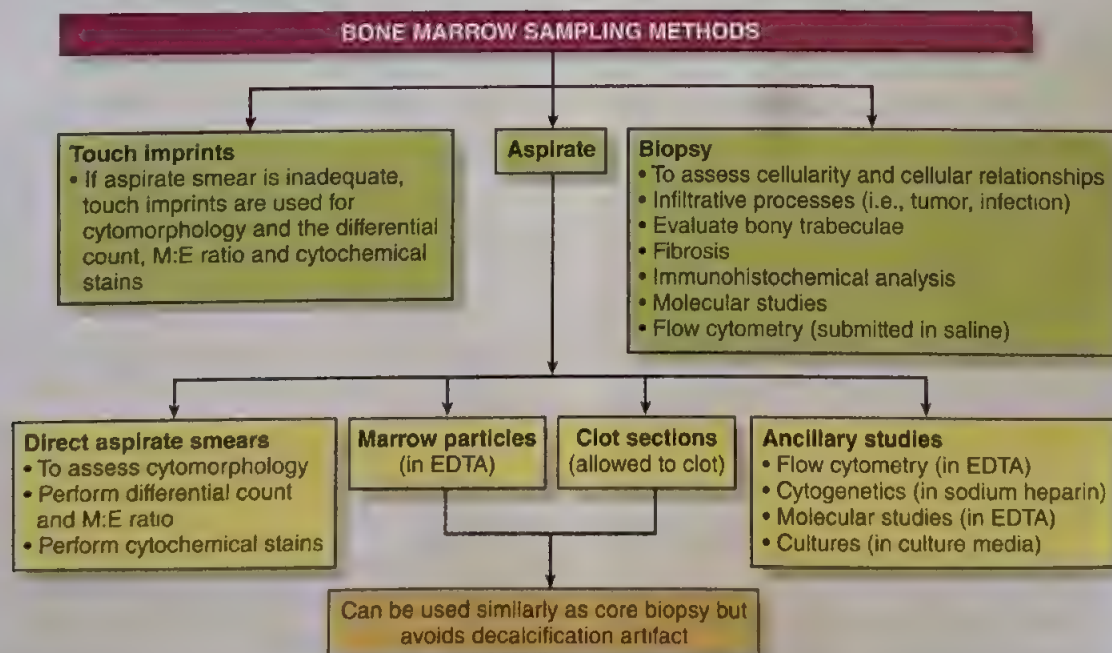


FIGURE 3-14 Distribution of bone marrow sample. EDTA = ethylene diaminetetraacetic acid; M:E = myeloid-to-erythroid ratio. (Note: Fluorescence in situ hybridization [FISH] studies can be performed on aspirate smears, touch preparations, and all of the tissue sections.)

may be obtained (placed in saline or RPMI – Roswell Park Memorial Institute medium) for flow cytometric and possibly other ancillary studies. In these situations, touch preparations of the core biopsy are useful for Wright-Giemsa-stained morphological evaluation, as well as cytochemical and fluorescence in situ hybridization (FISH) studies, if needed.

Preparation of Bone Marrow Aspirate

All necessary materials, preservatives, and slides should be meticulously clean and in readiness to avoid any delay. The aspirate in the first syringe contains mostly blood admixed with fat, marrow cells, and particles of marrow tissue, which should be used for smears. Several direct smears can be prepared immediately, using the technique for blood film preparation.²⁸ A small drop is placed on a glass slide, and the blood and particles are dragged behind a spreading slide with a technique similar to that for preparing blood films. Although this method of preparation preserves the cell morphology well, it is inadequate for the evaluation of the cells in relationship to each other and for the estimation of marrow cellularity.

Smears of marrow particles are prepared by pouring a small amount of the aspirate on a glass slide. The marrow tissue is seen as gray particles floating in blood and fat droplets. The particles are aspirated selectively with a plastic dropper or Oxford pipette and transferred to a clean glass slide, which is covered gently with another slide. The two slides are pulled in opposite and parallel directions to smear the particles without crushing the cells. Some people recommend an alternate technique using two cover slips. In this process, the marrow particles are squashed between two cover slips, which are then gently pulled apart.

Techniques for preparing particle smears vary from person to person and from laboratory to laboratory. The aspirate may be transferred into a watch glass and the particles collected with a capillary pipette or the broken end of a wooden stick applicator. With experience, one usually adapts a technique that facilitates production of high-quality slides. The medical laboratory scientist should prepare an adequate number of slides of smeared marrow particles. In cases of newly diagnosed acute leukemia, no fewer than 10 slides should be prepared. These are needed for histochemical stains such as myeloperoxidase, Sudan black B, naphthyl AS-D chloroacetate esterase, alpha-naphthyl butyrate esterase, iron, and others.³⁰

Marrow particle smears are used in the evaluation of cellularity (usually marrow biopsy is ideal) and the relationship of the cells to each other. Well-prepared smears have the added advantage of excellent cell morphology, allowing subtle changes in cell maturation and cytoplasmic inclusions to be recognized easily.

All direct and particle smears should be labeled at the bedside with the patient's name, identification number, and the date and then air-dried.

Histologic Marrow Particle Preparation

The leftover marrow particles obtained during the aspiration procedure can be processed for histologic examination. The tissue particles, admixed with blood, may be left to clot, then

fixed in 10% buffered formalin and processed for histologic sectioning. However, better results are obtained if the blood and particles are transferred to an EDTA anticoagulant-containing tube before clotting sets in. The blood and particles are then filtered through histo-wrap filter paper, and the concentrated particles enfolded in the paper are fixed in 10% buffered formalin. In the histology laboratory, these particles are collected by scraping the paper and then embedding the particles in paraffin for further processing.³⁰

Bone Marrow Core Biopsy

A bone marrow core biopsy is especially indicated when the marrow cannot be aspirated ("dry tap"), owing to pathological alterations encountered in acute leukemias, myelofibrosis, hairy cell leukemia, and other disorders. A trephine bone marrow core biopsy is also performed for the diagnosis of neoplastic and granulomatous diseases. In multiple myeloma and for staging of lymphomas or solid tumors, bilateral posterior superior iliac crest biopsies are recommended, as increased sampling size enhances the likelihood of capturing a focal process. An adequate biopsy sample is at least 15 mm in length.³¹

When a bone marrow biopsy is performed in conjunction with a marrow aspiration, customarily the biopsy sample is obtained after the aspirate. This sequence is usually achieved by changing the direction of the needle to avoid the aspiration artifact. However, this technique may result in an aspiration artifact with hemorrhage into the area of the biopsy site, leading to difficulties in evaluating cellularity and morphology (Figure 3-15). Therefore, a core biopsy sample should be obtained before the aspiration or the marrow biopsy procedure is to be performed through a new puncture site in the anesthetized area. In some cases when flow cytometry is indicated and the aspirate is difficult to obtain (e.g., in patients with hypercellular marrow, hairy cell leukemia, or marrow fibrosis), an additional core marrow biopsy may be obtained in normal saline or RPMI. Adequate cell suspensions can then be made from this biopsy sample and processed for flow cytometric studies.

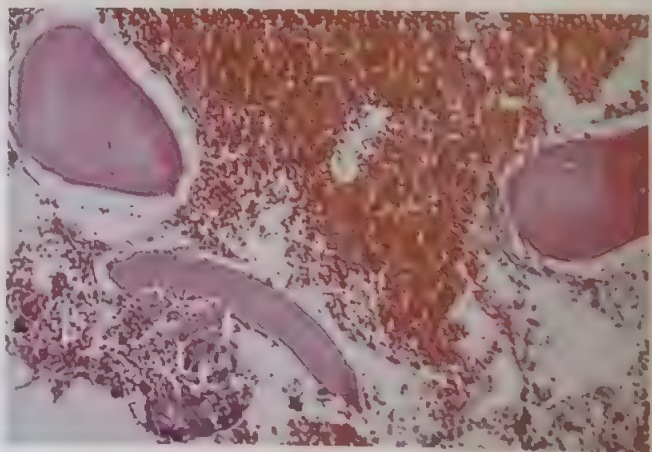


FIGURE 3-15 Bone marrow biopsy specimen showing aspiration artifact that can affect the cellularity and alter the relationship of cells to each other. (H&E, magnification $\times 200$)

Preparation of Trephine Biopsy

Touch Preparation

The bone marrow core biopsy sample is supported lightly without pressure between the blades of forceps and touched several times on two or three clean slide surfaces. The biopsy core sample should not be rubbed on the slide, because rubbing destroys the cells. The slides are air-dried. The touch preparations are fixed in absolute methanol and stained with Wright-Giemsa stain. In the absence of a good aspirate smear, the touch preparations may be the only source for studying cellular details and the maturation sequence of the bone marrow biopsy sample. For example, in a case of hairy cell leukemia, dry taps are common and cellular morphology by Wright-Giemsa-stained touch preparation may be a useful clue to the diagnosis and allow cytochemical staining procedures, such as tartrate-resistant acid phosphatase (TRAP) (Figs. 3-16 and 3-17). Sometimes the touch preparations contain enough cells to obtain differential counts and blast evaluation, and to perform histochemical studies.³⁰

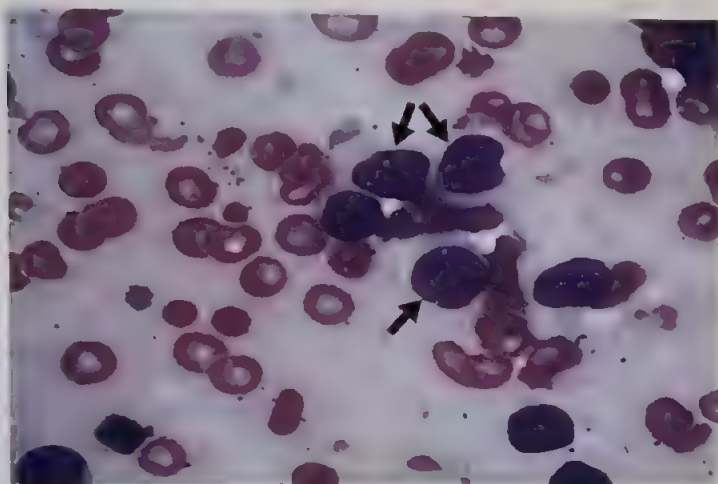


FIGURE 3-16 Wright-stained bone marrow touch preparation from a patient with hairy cell leukemia. Aspirate was a dry tap. A few diagnostic hairy cells are seen with abundant, fluffy, light blue cytoplasm and inconspicuous nucleoli. (magnification $\times 1,000$)



FIGURE 3-17 Tartrate-resistant acid phosphatase (TRAP) stain showing a strong positive reaction in neoplastic cells which is useful in the diagnosis of hairy cell leukemia. (magnification $\times 1,000$)

ADVANCED CONTENT

Histologic Bone Marrow Biopsy Preparation

The biopsy specimen is immersed without delay in B-S or 10% buffered formalin fixative. Histology laboratories may have a choice of other preferred fixatives such as Zenker's solution, Carnoy's solution, and others. After fixation, the biopsy specimen undergoes standard histologic processing of decalcification, dehydration, embedding in paraffin blocks, sectioning of 2- to 3- μ m thick sections, and histologic staining. The advantage of the bone marrow biopsy is that it represents a large sample of marrow and bone structures in their natural relationships. A variety of different stains can be used to demonstrate marrow iron, reticulum, and collagen. However, because of decalcification, the core biopsy may not be a good method for studying marrow iron stores, as the processing leaches iron from the tissue, which may be underrepresented in the iron stain. Acid-fast organisms and fungi in granulomatous diseases may be detected quickly with specific stains offering great advantages in diagnosing these infections (Figs. 3-18, 3-19, and 3-20). For example, mycobacterial cultures may require weeks of incubation to show growth of organisms, whereas on tissue sections, the histologic and etiological diagnosis may be made within 10 to 12 hours. When metastatic tumors and lymphomas are found in the bone marrow, immunohistochemical stains can be used on histologic sections to demonstrate specific tumor markers (Figs. 3-21, 3-22, and 3-23). Thus, a very precise diagnosis of the origin of a tumor can be made without elaborate expensive, and invasive techniques.

A disadvantage of the bone marrow biopsy is that fine cellular details are lost in the processing; therefore, it is of little value in the diagnosis of myelodysplastic syndromes and subtyping of acute leukemias. In these situations, the Wright-Giemsa-stained aspirates or core biopsy touch preparations may supply the missing morphological details.

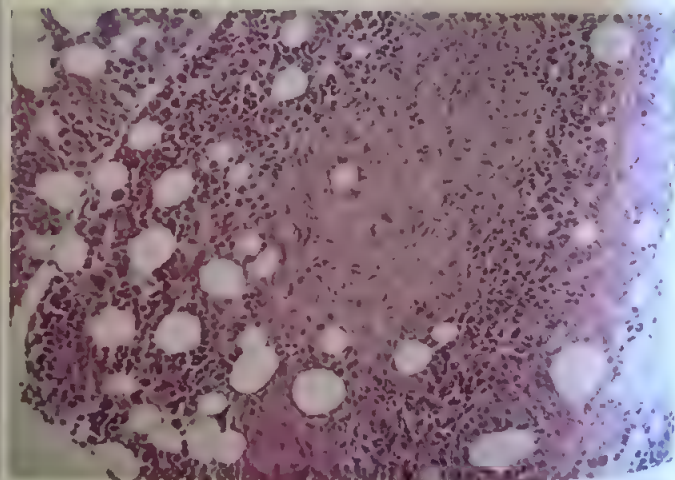


FIGURE 3-18 Bone marrow biopsy specimen from an HIV-positive patient with tuberculosis shows a well-formed granuloma. (H&E, magnification $\times 200$)

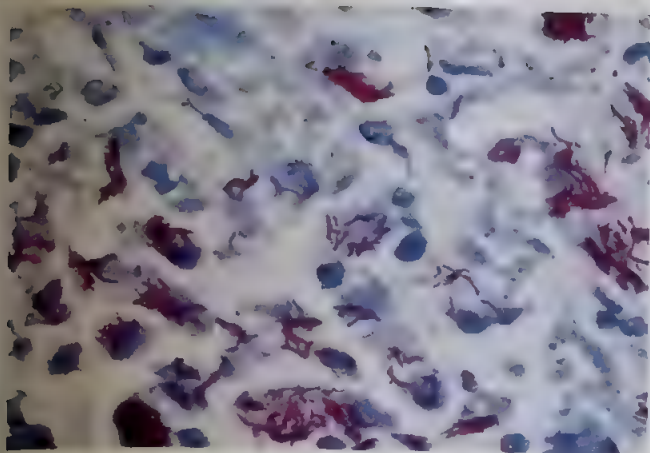


FIGURE 3-19 Acid-fast stain on a bone marrow biopsy specimen from the same patient as in Figure 2-18 shows acid-fast organisms, suggesting infection with *Mycobacterium*. (magnification $\times 1,000$)

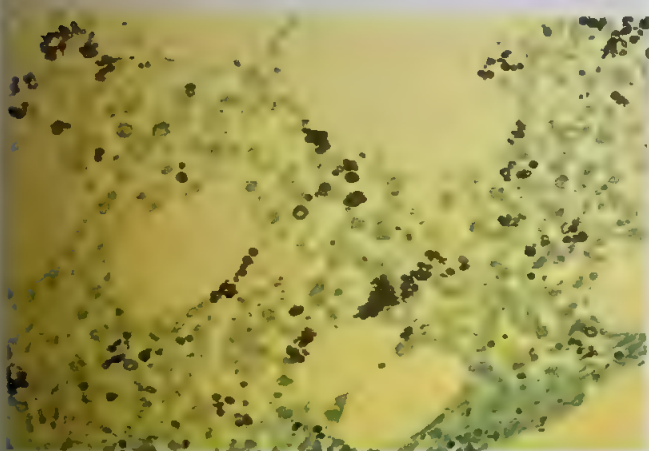


FIGURE 3-20 GMS (Gomori's methenamine silver) stain on bone marrow biopsy specimen from an HIV-positive patient shows multiple budding yeasts consistent with histoplasmosis. (magnification $\times 1,000$)

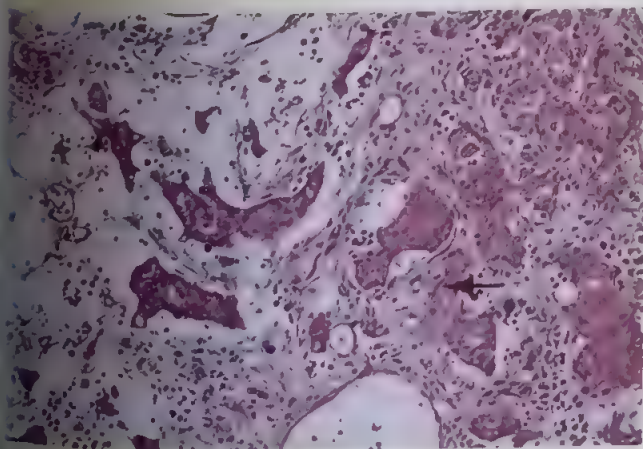


FIGURE 3-21 Bone marrow biopsy specimen from a patient with metastatic carcinoma shows glandular formation, a morphological feature of adenocarcinoma. (H&E, magnification $\times 600$)

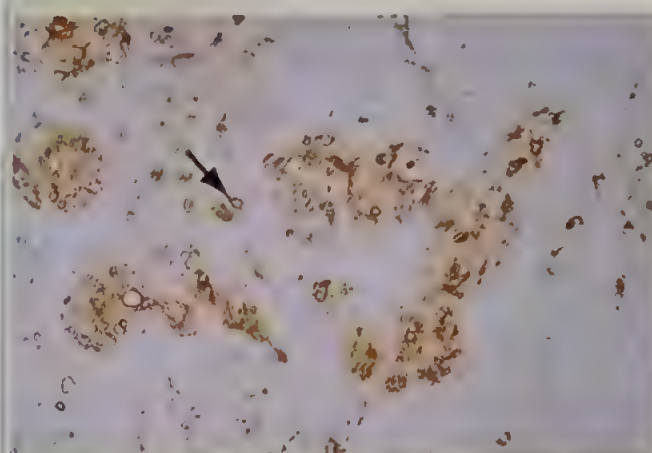


FIGURE 3-22 Immunohistochemical stain for prostate-specific antigen (PSA) performed on a marrow biopsy specimen from the same patient as in Figure 3-21. The specimen shows positive staining with PSA, thus confirming that the metastatic tumor is from prostate. (magnification $\times 600$)

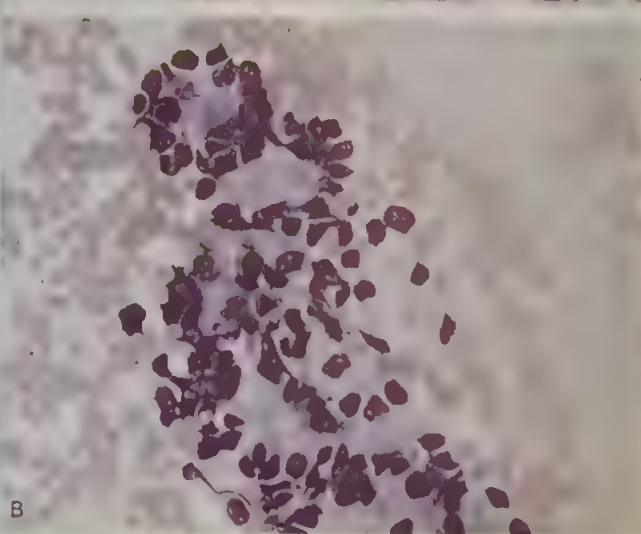
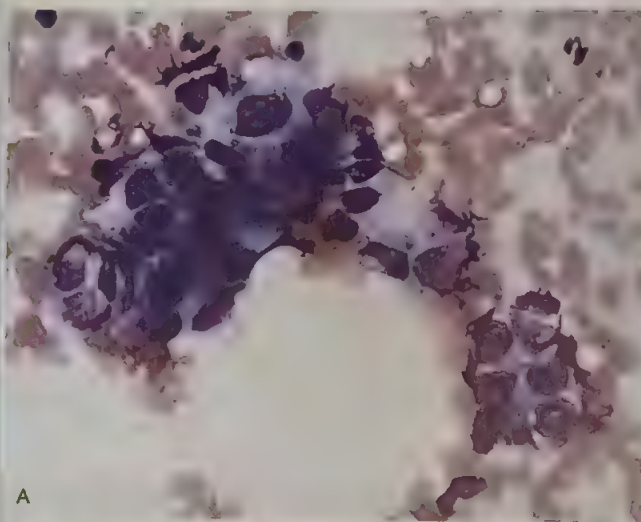


FIGURE 3-23 Aspirate smear from a patient with metastatic prostate carcinoma to the bone marrow. Note the cohesive crowded groups of large neoplastic cells (A) and glandular formation (B) suggesting adenocarcinoma. (Wright-Giemsa, magnification $\times 600$)

(Figs. 3–24 and 3–25). Multiple touch preparations also offer an opportunity for histochemical stains (myeloperoxidase, Sudan black B, naphthyl AS-D chloroacetate esterase, α -naphthyl butyrate esterase, etc.), which are essential in the classification of leukemias. Molecular genetics studies and FISH, though preferred on fresh tissue, can be performed on paraffin-embedded, formalin-fixed marrow biopsy specimens. Molecular testing may be used to evaluate B-cell or T-cell lymphomas, various leukemias, myelodysplastic syndromes, myeloproliferative neoplasms, and minimal residual disease.

Trephine bone marrow biopsy specimens may be embedded in methyl methacrylate, a synthetic plastic medium, and sectioned into 1- to 2- μ m thin sections without

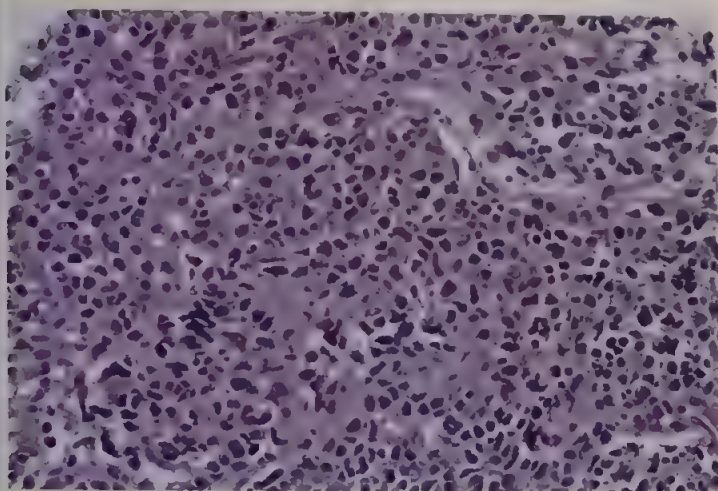


FIGURE 3-24 H&E-stained bone marrow biopsy specimen from a patient with acute lymphoblastic leukemia shows an increased number of blasts. Note that the blasts are not cohesive and are individually scattered. This is a morphological feature of hematolymphoid malignancy. Fine cellular details are lost in biopsy sections. (magnification $\times 600$)

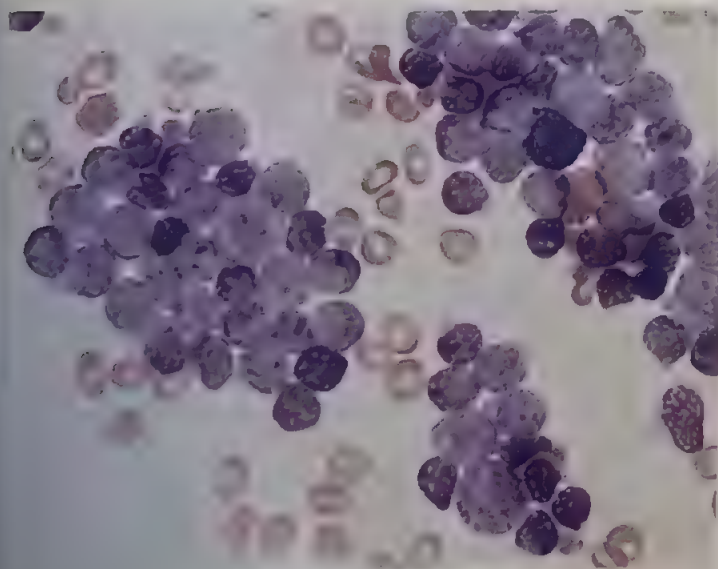


FIGURE 3-25 Wright-Giemsa-stained aspirate smear of acute lymphoblastic leukemia showing cytoplasmic and nuclear details useful in the diagnosis of acute leukemias. Compared with the metastatic tumor cells seen in Figure 3-23, the blasts are not cohesive and scattered.

decalcification. The morphological quality of the cells is extremely well preserved, and a differential count can be done on hematoxylin-eosin (H&E) or Giemsa-stained slides.^{28,30} However, this technique requires specially trained personnel, equipment, and separate handling in the histology laboratory, which increases the cost of the procedure. The processing time of the tissue also increases, which may not be acceptable if rapid diagnoses are required. In addition, tissue embedded in plastic media, instead of paraffin, may not be suitable for immunohistochemical studies of bone marrow.

CRITICAL THINKING QUESTION

3-2 Why is it so important for medical laboratory scientists to be at the bedside during a bone marrow procedure?

Bone Marrow Examination

The examination of the bone marrow aspirate smears should start at low magnification with a dry objective of 10 \times . Scanning the slide permits selection of a suitable area for examination and the differential count. “Bare nuclei” should be avoided; such nuclei result from destruction of the marrow cells by squashing or stripping of their cytoplasm by fibrin threads. An area is selected in which the cells are well spread, intact, and not diluted by sinusoidal blood. When marrow particles are examined, such areas are found at the periphery of the particles. At this low magnification, marrow cellularity is also evaluated. The megakaryocytes are usually noted adjacent to a spicule, about 5 to 10 per low-power field. Nonhematopoietic tumor cells infiltrating the bone marrow may also be seen at this magnification. These are usually larger than the granulocytic or erythropoietic precursors and are scattered in small groups and crowded clusters. Some show a glandular configuration (see Fig 3–23).

After the initial scan, immersion oil may be applied to the slide and the examination continues on high magnification (high dry or oil immersion objective 50 \times or 100 \times). The high magnification provides details of the nuclear and cytoplasmic maturation process (Fig. 3–26). The iron in histiocytes is visualized as brown-blue granules. Cytoplasmic inclusions of a diagnostic nature can be seen in histiocytes and granulocytes. Differential counts of bone marrow are performed under high magnification.

Estimation of Bone Marrow Cellularity

Cellularity is reflected in the ratio of nucleated hematopoietic cells to fat cells. Bone marrow cellularity normally varies with age, and the estimated cellularity must be compared with age-related normal ranges. At birth, the normal marrow cellularity is 100%. Thereafter, the cellularity gradually decreases. Overall marrow cellularity in adults is about 50% ($\pm 10\%$). The general rule to estimate age-related normal ranges is 100 minus age ± 10 . For example, the estimated normal marrow cellularity of a 40-year-old person would be 100–40 ± 10 (i.e., a range from 50% to 70%).

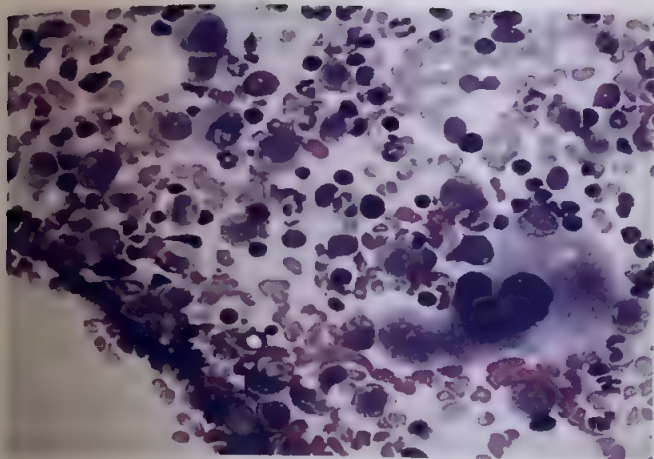


FIGURE 3-26 Smear of normal cellular marrow with normal maturation of erythropoietic, granulocytic, and megakaryocytic cells. (Wright-Giemsa, magnification $\times 200$)

Cellularity may vary from area to area, and, therefore, estimated cellularity should represent the average percentage. If hypercellular (90%) and hypocellular (10%) areas are seen, this finding should be mentioned descriptively in the report because, in such cases, an average cellularity may be difficult to estimate. The immediate subcortical region of adult bone marrow is usually hypocellular compared with the deeper medullary area. Therefore, sections that contain predominantly subcortical bone are frequently suboptimal for assessing true marrow cellularity.

The bone marrow biopsy specimen is most reliable for assessment of cellularity, because it offers a large amount of tissue for evaluation. However, the evaluation of cellularity can also be done on well-prepared aspirate smears or marrow particles. The best area for examination of cellularity in smears is the area between two uncrushed particles. The ratio of cells to fat is evaluated at low magnification (objective $10\times$), so that larger areas are included in the field of observation. The empty spaces that result from the spreading of the cells but are not occupied by fat cells are disregarded and treated as an artifact. The terms *decreased* or *increased cellularity* are used when fewer or more than the expected normal number of cells are found. Precise evaluation can be achieved with experience, and good reproducibility can be attained among several observers. The marrow cellularity can be expressed in percentages, but this is best done on histologic sections of biopsy specimens (Figs. 3-27, 3-28, and 3-29). Marrow cellularity has diagnostic value when it is related to the M:E ratio, which is calculated after a differential count is performed.

It is always important to look for any abnormal changes in the bony **trabeculae**. Various conditions can alter the morphological appearance of these trabeculae (Fig. 3-30). Marked thickening of trabeculae (Fig. 3-31) can be seen in myeloproliferative disorders (myelofibrosis with myeloid metaplasia), whereas thinning of trabeculae (Fig. 3-32) can be seen in older adults, in patients with acquired immunodeficiency syndrome (AIDS) or other cachectic conditions, and after chronic steroid administration. Various metabolic disorders can also alter the morphology of bony trabeculae. Examples include

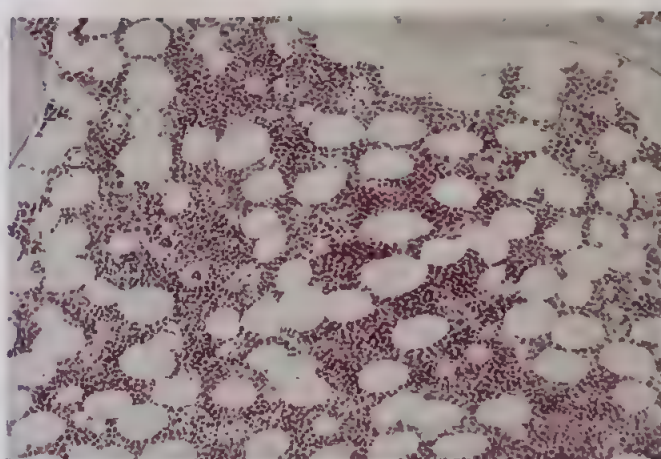


FIGURE 3-27 Normal bone marrow biopsy specimen from a 50-year-old patient shows approximately 50% cellularity. (H&E, low magnification)

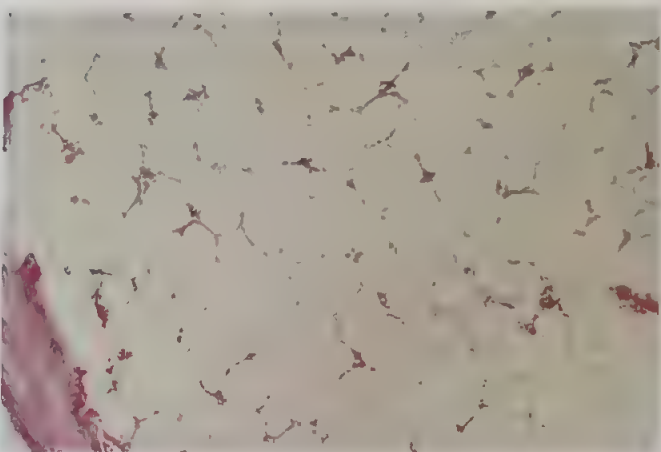


FIGURE 3-28 Markedly hypocellular bone marrow biopsy specimen from a 20-year-old patient. (H&E, low magnification)

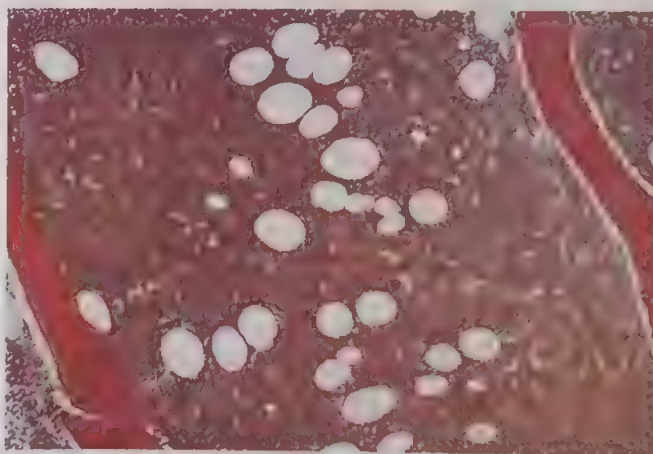


FIGURE 3-29 Hypercellular bone marrow biopsy specimen from a 60-year-old patient with 80% cellular marrow. This patient was receiving growth factor therapy. (H&E, low magnification)

a mosaic pattern, seen in patients with Paget's disease, and resorption and cyst formation, in persons with hyperparathyroidism and chronic renal failure.

When lymphoid aggregates are seen in the bone marrow biopsy specimen, the differential diagnosis includes benign

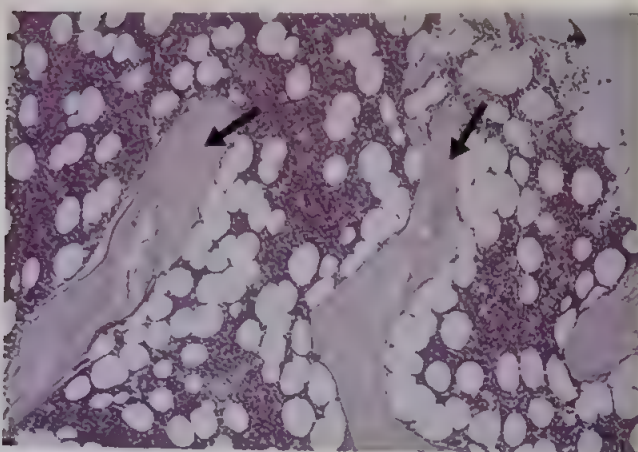


FIGURE 3-30 Bone marrow biopsy specimen showing normal bony trabeculae. (H&E, low magnification)



FIGURE 3-31 Bone marrow biopsy specimen shows marked thickening of bony trabeculae (osteosclerosis). (H&E, magnification $\times 200$)

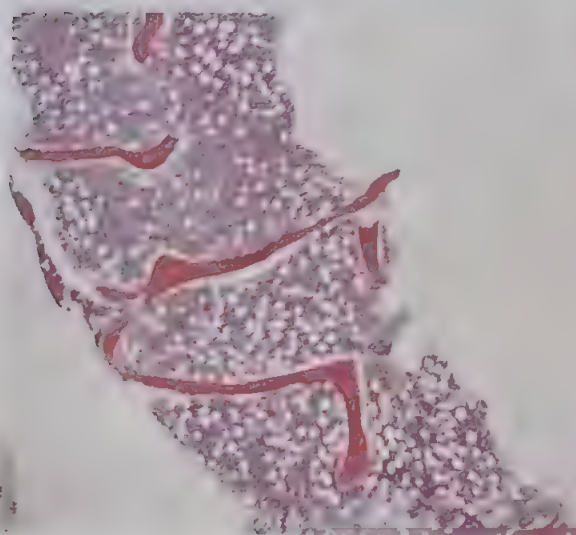


FIGURE 3-32 Bone marrow biopsy specimen shows thinning of bony trabeculae (osteopenia). (H&E, magnification $\times 200$)

lymphoid aggregates and malignant lymphoma. Usually benign aggregates are small and well demarcated, nonparatrabecular, and composed predominantly of small, round lymphocytes with plasma cells at the periphery and blood vessels present within the aggregate (see Figs. 3-5 and 3-33). Conversely, malignant follicles are usually large with ill-defined borders, paratrabecular, composed of atypical lymphocytes, and lack plasma cells at the periphery (Fig. 3-34). However, a neoplastic lymphoid infiltrate can also be interstitial, diffuse, and patchy. In some cases, immunohistochemical stains can be performed on the marrow core biopsy to differentiate between benign lymphoid aggregates and malignant lymphoma.

Bone marrow fibrosis may be found in patients with hairy cell leukemia, in myeloproliferative and myelodysplastic syndromes, sometimes in acute leukemia, after radiation, and after toxic injury to the marrow. On routine H&E sections streaming of marrow stroma and dilated sinusoids suggests

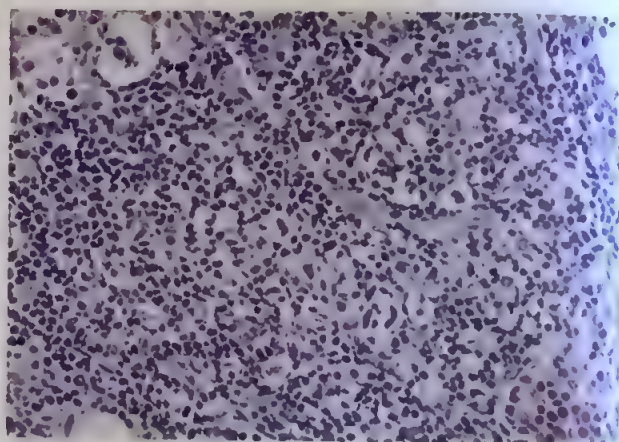


FIGURE 3-33 High-power view of the bone marrow biopsy from a 60-year-old patient shows lymphoid aggregate (low-power view is seen Fig. 3-5). Note the presence of blood vessel and plasma cells at the periphery of the lymphoid aggregate. (H&E, magnification $\times 400$)

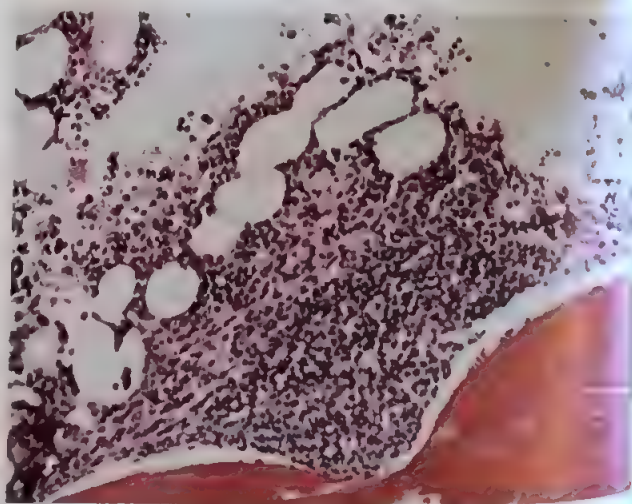


FIGURE 3-34 Bone marrow biopsy specimen shows a paratrabecular lymphoid aggregate. This pattern of infiltration is most likely indicative of involvement of the marrow by malignant lymphoma. (H&E, magnification $\times 200$)

marrow fibrosis; this finding can be confirmed and graded by performing reticulin and trichrome stains (Fig. 3-35). Normally, occasional reticulin-positive fibers may be seen around the blood vessels. The fibrosis can be graded as mild, moderate, or severe. In addition, a description of the fibrosis as fine or coarse, or focal or diffuse, may be helpful, especially when following the improvement of a patient with myelofibrosis after treatment or bone marrow transplantation. Trichrome stain is usually performed to detect any collagenous fibrosis, which if present may indicate irreversible fibrosis. Fibrosis and other bone marrow conditions such as marked hypercellularity in acute leukemias may result in a "dry tap" when attempting the aspiration procedure. However, if flow cytometry is needed, it is a good practice to obtain two bone marrow biopsy samples. One biopsy sample can be put in formalin and processed for morphological examination. The other core biopsy sample can be put in saline or RPMI medium; a cell suspension is then made for flow cytometric studies.

Bone Marrow Differential Count

A bone marrow differential count is an excellent tool for training a novice in bone marrow morphology and is widely used in diagnosing and following up patients with leukemias, refractory anemias, myelodysplastic and myeloproliferative neoplasms. Because of the compartmentalization of the hematopoietic cells and high cellularity of marrow, at least 500 to 1,000 nucleated cells need to be classified for a representative differential count.

In infants during the first month after birth, dramatic alterations occur in the distribution of the different marrow compartments. At birth, there is a predominance of granulocyte precursors, which switches within a month to a predominance of lymphoid elements. In early infancy, many lymphocytes have fine chromatin and a high nuclear-to-cytoplasmic ratio and lack distinct nucleoli.³¹ They are called hematogones and represent normal lymphoid progenitor cells.¹³ Hematogones may be misinterpreted as blasts if the observer is unfamiliar with these characteristics (see Fig. 3-6).³¹ In children up to 3 years old, one-third or more of the marrow cellularity is

made up of lymphocytes.³¹ The lymphocyte number gradually declines to the normal adult level thereafter.

In adult marrow, the lymphocytes are distributed randomly among the hematopoietic cells and within lymphoid follicles. This can introduce significant variation in the differential count from sample to sample in the same patient. Most adult marrow is composed of granulopoietic and erythropoietic precursors. For the differential count, these are enumerated into different categories according to their stage of maturation. When adequate numbers of cells are tabulated, the percentage of each category is calculated. The ratio between all granulocytes and their precursors and all nucleated red cell precursors represents the **M:E ratio**.

Some prefer to exclude the segmented neutrophils from the differential count as being part of the neutrophil storage pool of the marrow. The normal M:E ratio in this case is between 1.5 and 3. However, pathologists and hematologists who interpret the bone marrow histologic sections of particle clot and biopsies in conjunction with marrow smears include the segmented neutrophils in the differential counts, because these cannot be excluded in the evaluation of histologic specimens and are part of the marrow cellularity. The normal M:E ratio then is slightly higher and ranges between 2 and 4. The granulopoietic tissue occupies two to four times greater marrow space than the erythropoietic precursors, owing to the shorter survival of the granulocytes in the circulation (i.e., neutrophils, 6 to 10 hours, versus erythrocytes, 120 days). Changes in the survival of granulocytes and erythrocytes are reflected in changes in the M:E ratio.

Megakaryocytes are not included in the differential count. Megakaryocytes are unevenly distributed, and a differential count is a poor means for their evaluation. Usually, 5 to 10 megakaryocytes are seen per microscopic field at low magnification (objective 10 \times). When clusters of megakaryocytes and promegakaryocytes are seen in every field, it is an indication of megakaryocytic hyperplasia. In a normocellular marrow, finding fewer than two megakaryocytes per field on screening may indicate megakaryocytic hypoplasia. A marked increase or decrease in the number of megakaryocytes is easy to evaluate, whereas slight to moderate changes are difficult to judge and are better estimated on histologic sections of biopsy and particle specimens.

Table 3-2 represents the data of normal marrow reference ranges used by CorePath Laboratories at San Antonio, Texas.

Bone Marrow and Peripheral Blood Interpretation Based on Cellularity and M:E Ratio Changes

A bone marrow aspirate or biopsy sample represents a minute part of a very large and dynamic organ. Its activity and responses are reflected in blood changes; therefore, evaluation of the bone marrow should always be done in conjunction with evaluation of the peripheral blood. In adults with 50% marrow cellularity, about 30% to 40% represents granulopoiesis and 10% to 15% erythropoiesis, with an average M:E ratio of 4:1. An increase or a decrease in marrow cellularity with the normal M:E ratio usually indicates a balanced granulocytic and erythrocytic hyperplasia or hypoplasia, respectively. However, if cellularity changes occur simultaneously with the

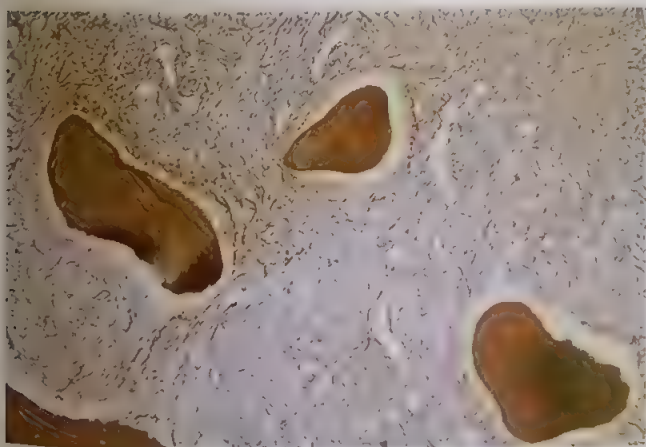


FIGURE 3-35 Reticulin stain on a marrow biopsy specimen from a patient with hairy cell leukemia (same patient as Figs. 3-16 and 3-17) showing diffuse and severe fibrosis. Dilated sinusoids are present. As expected, the aspirate was a dry tap. (magnification $\times 200$)

TABLE 3-2 Differential Cell Count of Bone Marrow in Percentage of Total Nucleated Cells*

Type of Cell	Reference Ranges			
	At Birth	Birth to 1 Month	Children	Adults
Undifferentiated Cells	0-2	0-2	0-1	0-1
Myeloblasts	0-2	0-2	0-2	0-2
Promyelocytes	0-4	0-4	0-4	0-4
Myelocytes				
Neutrophilic	2-8	2-4	5-15	5-20
Eosinophilic	0-5	0-3	0-6	0-3
Basophilic	0-1	0-1	0-1	0-1
Metamyelocytes and Bands				
Neutrophilic	15-25	5-10	5-15	5-35
Eosinophilic	0-5	1-5	1-8	0-5
Basophilic	0-1	0-1	0-1	0-1
Segmented Neutrophils	5-15	3-10	5-15	5-15
Pronormoblasts	0-3	0-1	0-2	0-1.5
Basophilic Normoblasts	0-5	0-3	0-5	0-5
Polychromatophilic Normoblasts	6-20	5-50	5-11	5-30
Orthochromatic Normoblasts	0-5	0-2	0-8	5-10
Lymphocytes	5-15	5-20	5-35	10-20
Plasma cells	0-2	0-2	0-2	0-2
Monocytes	0-2	0-2	0-2	0-5

*Normal reference ranges from CorePath Laboratories, San Antonio, TX.

M:E ratio change, the interpretation requires a broader understanding of hematopoietic tissue physiology and its reactions during disease.

Cell morphology and the M:E ratio are well represented in random bone marrow specimens. The variations are not significant even when samples are compared from sternal and iliac crest aspirates. However, marrow cellularity is poorly represented in random smears; thus, this interpretation should be considered with some degree of reservation. Even large biopsy specimens may have a great degree of variation in cellularity. For these reasons, in diseases in which marrow cellularity is crucial for the diagnosis (aplastic anemia, marrow hypoplasia), more than one bone core biopsy may be required.

The Marrow and Blood Interpretation Based on Cellularity and M:E Ratio (Table 3-3) has been included to provide both a simple guide and some basic information to the reader. It cannot serve as a diagnostic tool without the addition of the patient's clinical history and a clinical evaluation of the disease. The reader is also cautioned that the variety of problems frequently presented by different patients with the same disease may not fit within such a simple schematic concept.

Bone Marrow Iron Stores

The storage iron of the bone marrow is in the form of **hemosiderin**. The iron content of hemosiderin is higher than that of ferritin. Other components of hemosiderin are protein, ferritin aggregates, some lipids, and membranes of cellular organelles. Hemosiderin can be seen on unstained smears as golden-yellow granules. On Wright-Giemsa-stained smears it appears as brownish-blue granules. However, for more precise evaluation, Prussian blue reaction is used to demonstrate the intracytoplasmic iron of histiocytes and red cell precursors. The evaluation of marrow iron stores is essential in the diagnosis of anemias and especially in refractory and dyserythropoietic anemias. When the morphological characteristics of the iron particles in the storage nutrient histiocyte and erythroblastic precursors is an important diagnostic consideration (e.g., in sideroblastic anemias), an iron stain is performed on a particle smear. If the overall distribution of the amount of iron is of clinical importance (e.g., iron-deficiency anemia, anemia of chronic inflammation (ACI), hemochromatosis, and others), then histologic sections of bone marrow biopsy sample, and/or marrow aspirate, and marrow clotted particles are stained for iron. The biopsy sample and the particles are a more reliable source of information, because they

TABLE 3-3 Marrow and Blood Interpretation Based on Cellularity and M:E Ratio

Complete Blood Count	Bone Marrow Cellularity	M:E Ratio	Bone Marrow Interpretation
Normal	Increased or decreased*	Normal	Normal
Neutropenia	Decreased	Decreased	Granulocytic hypoplasia
Neutropenia	Normal or increased	Increased	Decreased neutrophilic survival or ineffective granulopoiesis
Neutrophilia	Normal or increased	Increased	Granulocytic hyperplasia
Anemia	Normal or decreased	Increased	Red cell hypoplasia
Anemia	Normal or increased	Decreased	Erythrocytic hyperplasia or ineffective erythropoiesis†
Erythrocytosis	Normal or increased	Decreased	Erythrocytic hyperplasia (polycythemia)
Pancytopenia	Decreased	Normal	Marrow hypoplasia
Pancytopenia	Increased	Normal, increased, or decreased	Ineffective myelopoiesis or hypersplenism

*Because of poor representation of cellularity in random specimen.

†Reticulocyte count is necessary to differentiate between erythrocytic hyperplasia and ineffective erythropoiesis.

represent a large sample of hematopoietic tissue. Bone marrow biopsy samples for iron studies should be decalcified by the EDTA chelating method, which does not affect the storage iron. Rapid-acid decalcifying solutions extract iron and must not be used in these cases.

After Prussian blue staining, hemosiderin and some ferritin aggregates are seen as bright blue specks and granules (Fig. 3-36). Hemoglobin iron and dispersed ferritin do not stain. Normal marrow iron is seen as fine cytoplasmic granules within histiocytes, and 30% to 50% of marrow erythroblasts contain iron specks within mitochondria and are called **sideroblasts**. Clumps of iron easily seen at scanning magnification (10×) indicate increased iron storage, whereas only a few specks of iron found after searching several microscopic fields (50× or 100× magnification) indicates decreased iron storage. When no stainable iron is detected on the bone marrow smear or tissue sections, this indicates iron storage depletion or absence. The storage iron may be reported as “absent,”

“decreased,” “adequate,” “moderately increased,” and “markedly increased,” or it can be given corresponding numerical values from 0 to 4, where 2 represents the normal or adequate iron stored in an adult. In children, however, iron is stored mainly in ferritin and does not stain with Prussian blue in normal iron states. Some chronic derangements in iron metabolism (i.e., myelodysplastic syndromes) may result in aberrant ringed sideroblastic iron around erythroblast nuclei; these cells are named ringed sideroblasts.

CRITICAL THINKING QUESTION

3-3 What is the significance of the Prussian blue stain?

Bone Marrow Report

The bone marrow report usually encompasses the following information:

1. The name of the laboratory or physician's office from which the report originates.
2. The patient's data, including age, unique specimen identifier, and relevant clinical summary or clinical diagnosis such as recent chemo/radiotherapy or cytokine treatments.
3. A description of material received for studies, such as smears of aspirate, marrow particles, and bone biopsy (or biopsies).
4. Data from the complete blood count (CBC) and WBC differential count, and a description of the blood smear, preferably from the day on which the bone marrow specimen is obtained. A platelet count should be included, as well as a reticulocyte count, if available.
5. The bone marrow differential count.
6. A description of cellularity, M:E ratio, granulopoiesis, erythropoiesis, and megakaryocytopoiesis. Any change in the nonhematopoietic elements of marrow, such as hemophagocytosis, granulomas, microorganisms, metastatic tumor cells, histiocytic hyperplasia, or the

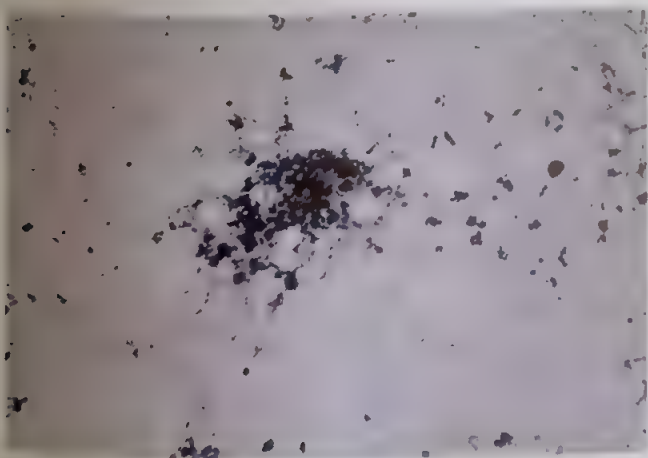


FIGURE 3-36 Prussian blue iron stain of a bone marrow smear shows a marked increase in marrow storage iron, thus ruling out the possibility of iron-deficiency anemia. (magnification ×200)

appearance of bony trabeculae, is included in this section of the report. The status of iron stores and special staining procedures performed are reported.

7. A description of histologic sections of marrow particles or bone marrow biopsy.
8. Cytochemistry or other investigative testing such as flow cytometry, molecular genetics FISH, cytogenetics, or microbiology.
9. The diagnostic conclusion. This should encompass separate diagnoses of blood and bone marrow even where the same diagnosis is applicable to both; for example: *Blood*—pancytopenia, *Bone marrow, left posterior iliac spine aspirate and biopsy*—myelodysplastic syndrome, refractory anemia with ringed sideroblasts; or *Blood*—acute myeloid leukemia minimally differentiated (WHO classification), *Bone marrow, left posterior iliac crest aspirate and biopsy*—acute myeloid leukemia minimally differentiated (WHO classification).

In summary, bone marrow can provide a representative picture of disease processes and has wide application in clinical medicine. Marrow examination has a significant role in the evaluation of leukemias, lymphomas, plasma cell disorders, myeloproliferative disorders, myelodysplastic disorders, myelofibrosis, metastatic tumors, various anemias, granulomatous diseases, infectious diseases, and metabolic diseases. In evaluating the status of engraftment after bone marrow transplantation; and in assessing chemotherapy effects. The medical laboratory scientist's contribution in this phase consists of preparing the optimum blood and bone marrow slides and performing the differential count. Examination of the blood and bone marrow, correlation with the clinical presentation, and diagnostic conclusions on each specimen are the responsibility of a physician who has adequate training and experience to integrate all the available clinical and laboratory information in reaching the correct diagnosis.

SUMMARY CHART

- The hematopoietic system consists of bone marrow, liver, spleen, lymph nodes, and thymus. In normal adults, hematopoiesis occurs mainly in the bone marrow.
- The bone marrow is one of the body's largest organs, representing 3.4% to 6% of total body weight and averaging about 1,500 g in adults.
- The structure of the bone marrow consists of hematopoietic cells (erythroid, myeloid, lymphoid, and megakaryocytes), adipose tissue, bone and its cells (osteoblasts and osteoclasts), and stroma.
- Erythropoiesis takes place in distinct anatomic units called erythropoietic islands.
- Granulocytic precursors are located deep in the hematopoietic cords and around the bone trabeculae.
- Megakaryopoiesis occurs adjacent to the sinus endothelium; megakaryocytes protrude as small cytoplasmic processes through the vascular wall, delivering platelets directly to the sinusoidal blood.
- Lymphocyte production is compartmentalized in lymphoid follicles, and lymphocytes are randomly dispersed throughout the hematopoietic cords.
- Hematogones are normal cellular constituents of bone marrow that resemble small-to-intermediate sized lymphocytes; they range in size from 10 to 20 μm , have a high N:C ratio and deeply basophilic cytoplasm, and are devoid of any granules or vacuoles.
- The meshwork of stromal cells in which the hematopoietic cells are suspended is in a delicate semifluid state and is composed of reticulum cells, histiocytes, fat cells, and endothelial cells.
- Among the most common indicators for bone marrow studies are diseases that affect the bone marrow, causing a decrease or increase in any of the cellular blood elements.
- In adults, the most common site for bone marrow aspiration or biopsy is the posterior superior iliac crest; in newborns and infants, bone marrow is obtained from the upper end of the tibia.
- In a bone marrow aspiration, 1.0 to 1.5 mL of marrow particles and sinusoidal blood is drawn into a syringe; all direct smears should be prepared quickly from unclotted specimens and labeled at the bedside with the patient's name, identification number, and date.
- When marrow cannot be aspirated ("dry tap"), the common differential diagnosis includes acute leukemia, myelofibrosis, and hairy cell leukemia.
- In the estimation of marrow cellularity (ratio of nucleated hematopoietic cells to fat cells) low-power magnification is used. Overall marrow cellularity in adults is about $50\% \pm 10\%$.
- When performing the marrow differential count, at least 500 to 1,000 nucleated cells are classified; the oil immersion objective is used, and megakaryocytes are not included in the differential count.
- The normal M:E (myeloid-to-erythroid) ratio for adults is 4:1; granulopoietic tissue occupies a marrow space that is two to four times greater than that occupied by the erythropoietic precursors.
- The storage form of iron in the bone marrow is hemosiderin. On Wright-stained smears, iron appears as brownish-blue granules. Decalcification leaches iron from the tissue and may result in an erroneous interpretation of absent iron stores in the core biopsy.

CASE STUDY 3-1**PATIENT NAME:** John Doe**PATHOLOGY SERVICES, UNIVERSITY HEALTH SYSTEM****HOSPITAL NUMBER:** XXXXXX**SURGICAL PATHOLOGY ACCESSION NUMBER:** S00-00000**PHYSICIAN:** Dr. Z**BONE MARROW EXAMINATION GROSS DESCRIPTION**

Specimen A: Specimen label, patient's name, and right posterior iliac crest biopsy. Received in formalin is a 1.2 cm × 0.2 cm × 0.2 cm bone marrow biopsy sample. Entirely submitted after decalcification: block A.

Specimen B: Specimen label, patient's name, and right posterior iliac crest aspirate. Received in EDTA are marrow particles that aggregate to 0.8 cm × 0.5 cm × 0.2 cm. Entirely submitted: block B.

Aspirate smears: 14 unstained and 4 Wright-stained samples from the right posterior iliac crest, all labeled with the patient's name. Touch preparations: 3 unstained and 2 Wright-stained samples from the right posterior iliac crest, all labeled with patient's name.

Also received is bone marrow aspirate in one EDTA tube for flow cytometry and one sodium heparin tube for cytogenetic studies.

MICROSCOPIC DESCRIPTION**Peripheral Blood**

CBC obtained from University Hospital on 02/22/06 reveals:

RBC: $2.5 \times 10^{12}/L$	WBC: $20 \times 10^9/L$
Hgb: 7.5 g/dL	Manual differential
Hct: 22%	Segmented neutrophils: 31%
MCV: 94.0 fL	Bands: 1%
MCH: 28 pg	Lymphocytes: 20%
MCHC: 32 g/dL	Monocytes: 2%
RDW: 19.0	Eosinophils: 1%
Platelet count $5.0 \times 10^9/L$	Basophils: 1%
	Metamyelocytes: 4%
	Myelocytes: 3%
	Promyelocytes: 2%
	Blasts: 35%

Anisocytosis: moderate with occasional microcytes and few macrocytes.

Poikilocytosis: mild with a few teardrop cells and rare schistocytes.

Polychromasia: mild. NRBCs are present 2/100 WBCs

The white blood cells display a left shift with increased number of circulating blasts. The blasts are of small size, show minimal variation, and have high nuclear-to-cytoplasmic ratio and fine and lacy chromatin with occasional small nucleoli. No Auer rods are seen.

The platelet count is $5.0 \times 10^9/L$. Few giant platelets are seen. No platelet clumps are noted.

Bone Marrow

Examination of the bone marrow aspirate smears reveals hypercellular marrow with decreased megakaryocytes. Differential count performed on aspirate smears reveals:

Blasts: 65%	Pronormoblasts: 1%
Promyelocytes: 6%	Basophilic normoblasts: 4%
Myelocytes: 5%	Polychromatic normoblasts: 4%
Metamyelocytes and bands: 4%	Orthochromatic normoblasts: 7%
Segmented neutrophils: 2%	Monocytes: 1%
	Lymphocytes: 1%

Blasts are markedly increased. The morphology of blasts is similar to that described in the peripheral smear. Residual myeloid and erythroid precursors showing progressive maturation are seen in the background. The M:E ratio is 5:1.

Cytochemical stains performed on the aspirate smears show that the blasts are negative for Sudan black B, myeloperoxidase, specific and nonspecific esterase, and periodic acid-Schiff (PAS).

Sections from the right iliac crest biopsy and right particle preparation reveal hypercellular marrow (cellularity 90%) with complete involvement by leukemic blasts. Scant residual trilineage hematopoiesis is seen in the background. Bony trabeculae are unremarkable.

DIAGNOSIS**Blood:**

Acute lymphoblastic leukemia, precursor B-cell (circulating blasts 35%)

Normochromic normocytic anemia, moderate

Thrombocytopenia, marked

Bone Marrow, Right Iliac Crest Aspirate Smears, Aspirate Particle Preparation, and Biopsy:

Acute lymphoblastic leukemia, precursor B-cell (blasts 65%, cellularity 95%) (see comment).

COMMENT

Flow cytometry performed on the bone marrow aspirate reveals a precursor B-cell phenotype. The blasts have the following phenotype: CD19, CD10, CD34, CD22, HLA-DR positive; and CD20, kappa (κ , lambda (λ) light chain negative. Other T-cell and myelomonocytic markers are negative. Cytogenetic studies are pending, and an amended report will follow. A call was made, and results were given to the patient's physician.

QUESTIONS

1. Based on the initial CBC report, what conditions are preliminary indicated?
2. Were the conditions right for a bone marrow study?

ANSWERS:

1. The low RBC count could indicate anemia. The low platelet count indicates thrombocytopenia. The WBC

Continued

CASE STUDY 3-1—cont'd

count is increased, and the differential reveals immature cells, which could indicate a malignant cell process.

2. Yes, this patient has increases and decreases in multiple cell lines that need to be evaluated at the bone marrow

level. Additionally, diagnosis cannot be made without histological review and flow cytometry studies in correlation with the peripheral findings.

CASE STUDY 3-2

PATIENT NAME: Jane Doe

PATHOLOGY SERVICES, UNIVERSITY HEALTH SYSTEM

HOSPITAL NUMBER: XXXXXX

SURGICAL PATHOLOGY ACCESSION NUMBER: S00-00000

PHYSICIAN: Dr. Y

HISTORY: 65-year-old woman with persistent pancytopenia and normal vitamin B₁₂ and folate levels.

BONE MARROW EXAMINATION GROSS DESCRIPTION

Specimen A: Specimen labeled with patient's name and right posterior iliac crest biopsy. Received in formalin is a 1.2 cm × 0.2 cm × 0.2 cm bone marrow biopsy sample. Entirely submitted after decalcification: block A.

Specimen B: Specimen labeled with patient's name and right posterior iliac crest aspirate. Received in EDTA are marrow particles that aggregate to 0.8 cm × 0.5 cm × 0.2 cm. Entirely submitted: block B.

Aspirate smears: 14 unstained and 4 Wright-stained samples from the right posterior iliac crest, all labeled with the patient's name. Touch preparations: 3 unstained and 2 Wright-stained samples from the right posterior iliac crest, all labeled with patient's name.

Also received is additional bone marrow aspirate in one sodium heparin tube for cytogenetic studies.

MICROSCOPIC DESCRIPTION**Peripheral Blood**

CBC obtained from University Hospital on 06/22/06 reveals:

RBC: $3.0 \times 10^{12}/L$	WBC: $2 \times 10^9/L$
Hgb: 9.0 g/dL	Manual differential
Hct: 27%	Segmented neutrophils: 55%
MCV: 102 fL	Blasts 2%
MCH: 28 pg	Lymphocytes: 30%
MCHC: 32 g/dL	Monocytes: 10%
RDW: 19.0	Eosinophils: 2%
Platelet count $10.0 \times 10^9/L$	Basophils: 1%

There is moderate aniso-poikilocytosis with many macrocytic red cells and few target cells. Coarse basophilic stippling is present.

Polychromasia: minimal

The white blood cells display few circulating blasts (2%). No hypersegmented neutrophils are seen. The platelet count

is $10.0 \times 10^9/L$. Few large and hypogranular platelets are seen. No platelet clumps are noted.

Bone Marrow

Examination of the bone marrow aspirate smear reveals hypercellular marrow. Differential count performed on aspirate smears:

Blasts: 1%	Pronormoblasts: 12%
Promyelocytes: 8%	Basophilic normoblasts: 25%
Myelocytes: 9%	Polychromatic normoblasts: 16%
Metamyelocytes and bands: 4%	Orthochromatic normoblasts: 7%
Segmented neutrophils: 10%	Monocytes: 4%
	Lymphocytes: 4%

The M:E ratio is 0.5:1. There is an increase in erythroid precursors with moderate dyserythropoiesis characterized by megaloblastoid changes, nuclear irregularity, binucleation, and nuclear fragmentation. Megakaryocytes are increased. Myeloid precursors are decreased in number and show progressive maturation to the neutrophil stage. Only a few blasts are present, and no Auer rods are seen in their cytoplasm.

An iron stain performed on the aspirate smear shows increased storage iron with many ringed sideroblasts.

Sections from the right iliac crest biopsy and right particle preparation reveal hypercellular marrow (cellularity 90%) with increased erythroid precursors. Aggregates of blasts are not seen. No lymphoid aggregates or granulomata are seen. Bony trabeculae are unremarkable. Reticulin stain of the core biopsy did not show increased fibrosis.

DIAGNOSIS**Blood:**

Pancytopenia with macrocytic anemia (circulating blasts 2%)

Bone Marrow, Right Iliac Crest Aspirate Smears, Aspirate Particle Preparation, and Biopsy:

Myelodysplastic syndrome, refractory anemia with ringed sideroblasts (cellularity 90%; blasts 1%) (see comment).

COMMENT

The patient's serum B₁₂ and red cell/serum folate levels are normal. The presence of pancytopenia in conjunction with a hypercellular marrow, red cell dysplasia, and many ringed

CASE STUDY 3-2—cont'd

sideroblasts is supportive of a diagnosis of a myelodysplastic syndrome, refractory anemia with ringed sideroblasts subtype. Preliminary cytogenetic studies performed on the marrow aspirate reveal clonal chromosomal abnormalities including monosomy 7, del(7q). Results called to Dr. Y at University Hospital.

QUESTIONS

1. Were bone marrow studies indicated in this patient?

2. Because there were iron evaluations in the bone marrow study, which stains were likely utilized?

ANSWERS

1. Yes, the patient had persistent pancytopenia, which is decreased in all cell lines.
2. Prussian blue was likely used to see the hemosiderin stores and the sideroblasts more clearly.

REVIEW QUESTIONS

1. The average life spans of red cells, neutrophils, and platelets in the circulation are:
 - a. 120 days, 7 days, and 10 days
 - b. 30 days, 7 days, and 10 days
 - c. 120 days, 6–10 hours, and 10 days
 - d. 30 days, 6–10 hours, and 10 hours
2. Bone marrow aspirate smears are more informative than the bone core biopsy in the diagnosis and classification of:
 - a. Granulomatous diseases
 - b. Acute leukemias
 - c. Metastatic carcinomas
 - d. Gaucher's disease
3. The least reliable specimen on which to perform an iron stain is:
 - a. Core biopsy
 - b. Clot section
 - c. Aspirate smears
 - d. Particle preparation
4. Marrow stem cells are capable of which of the following?
 - a. Differentiating into any cellular line
 - b. Producing antibodies
 - c. Stimulating the hemostasis system
 - d. Initiating the complement system
5. If the aspirate is unsuccessful (i.e., a "dry tap"), which alternative processing steps are possible?
 - a. Touch preparations of the core biopsy can be used for CBC test
 - b. Touch preparations of the core biopsy can be used for Wright–Giemsa morphological evaluation
 - c. Touch preparations of the core biopsy can be used for Coagulation tests
 - d. Touch preparations of the core biopsy can be used for gram staining
6. The bone marrow's cellularity is found in the:
 - a. Yellow marrow
 - b. Cartilage
 - c. Red marrow
 - d. Spleen
7. Which of the following statements describes hematogones?
 - a. They are malignant cells.
 - b. They belong to the granulocytic series.
 - c. They morphologically mimic blasts of lymphoblastic leukemia.
 - d. They are most commonly seen in elderly individuals.
8. Which is an example of antigen-independent proliferation?
 - a. The proliferation of plasma cells
 - b. The proliferation of NK cells
 - c. The proliferation of a myeloblast
 - d. T cells stimulating the proliferation of immune cells
9. The most appropriate site for bone marrow studies in adults is:
 - a. Anterior superior iliac crest
 - b. Posterior superior iliac crest
 - c. Sternum
 - d. Tibia
10. The production of cellular components and response to increased need for cell lines is the main role of which structure?
 - a. Liver
 - b. Adrenal glands
 - c. Bone marrow
 - d. Spleen

Continued

REVIEW QUESTIONS—cont'd

11. Adult bone marrow should have a cellularity of which percentage?
 - a. 100%
 - b. 75%
 - c. 60%
 - d. 50%
12. Which cellular components represent the M:E ratio?
 - a. Myeloid:Megakaryocytes
 - b. Myeloid:Erythroid
 - c. Megakaryocytes:Erythroid
 - d. Monoblasts:Eosinophils
13. Hematopoiesis is:
 - a. The process of red blood cell production
 - b. The process of white blood cell production
 - c. The production and maturation of blood cells
 - d. The differentiation and maturation of granulocytes

See answers at the back of this book.

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Examination of the Peripheral Smear

Red Cell, White Cell, and Platelet Morphology

Leslie A. Cooper, MS, MLS(ASCP)^{CM}, AHI(AMT)

CHAPTER OUTLINE

Automation in the Hematology Laboratory

Examination of the Peripheral Blood Smear

Low-Power (10×) Scan

High-Power (40×) Scan

Oil Immersion (100×) Examination

The Normal Red Blood Cell

Assessment of Red Cell Abnormality

Variations in Red Cell Distribution

Normal Distribution

Abnormal Distribution

Variations in Red Cell Size

Anisocytosis

Normocytes

Macrocytes

Microcytes

Hemoglobin Content—Red Cell Color

Variations

Normochromia

Hypochromia

Hyperchromia

Polychromasia

Variations in Red Cell Shape

Poikilocytosis

Target Cells (Codocytes)

Spherocytes

Stomatocytes

Ovalocytes and Elliptocytes

Sickle Cells (Drepanocytes)

Fragmented Cells

Burr Cells (Echinocytes)

Acanthocytes (Thorn Cells, Spur Cells)

Teardrop Cells (Dacrocytes)

Red Cell Inclusions

Howell–Jolly Bodies

Basophilic Stippling

Pappenheimer Bodies and Siderotic

Granules

Heinz Bodies

Cabot Rings

Hemoglobin CC Crystals

Hemoglobin SC Crystals

Protozoan Inclusions

Examination of Platelet Morphology

Examination of White Blood Cell Morphology

Immature White Blood Cells

White Blood Cell Morphology

WBC Cytoplasmic Inclusions

Summary Chart

Case Study 4–1

Case Study 4–2

Review Questions

References

LEARNING OBJECTIVES

At the end of the chapter, the learner should be able to:

- 4-1 Define the terms **flagged**, **delta check**, and **reflex test** as they pertain to automated hematology results.
- 4-2 List criteria for performing a manual morphology review.
- 4-3 Describe the steps involved in the examination of a stained peripheral blood smear.
- 4-4 Detail the key characteristics of a red blood cell with normal morphology on the peripheral smear.
- 4-5 Correlate variation in red blood cell shape, size, or color with the appropriate red blood cell indices reported from automated findings.
- 4-6 Define **anisocytosis** and **poikilocytosis**.
- 4-7 Contrast normal red blood cell distribution from abnormal distribution patterns.
- 4-8 State the **microcytic**, **macrocytic**, **normochromic**, **hypochromic**, and **hyperchromic** findings to their corresponding red blood cell indices and probable anemic states.

- 4-9 Describe **target cells**, **spherocytes**, **ovalocytes**, **elliptocytes**, **stomatocytes**, **drepanocytes**, **echinocytes**, and **dacrocytes** on a peripheral smear.
- 4-10 Correlate abnormal red blood cell shapes with their pathophysiologies and diseases in which they are often seen.
- 4-11 Describe the most common inclusions found in red blood cells, including their composition and the clinical conditions in which they may be seen.
- 4-12 Contrast normal platelet morphology with abnormal platelet morphologies.
- 4-13 Identify all five white blood cells on a peripheral smear.
- 4-14 Identify changes in leukocyte morphology, including presence of cytoplasmic inclusions and nuclear changes when presented with various clinical conditions.

Hematology is a largely automated discipline; however, the foundational knowledge of blood cell morphology remains a crucial part of both comprehending this material and providing accurate results. Advancements in multichannel automated hematology analyzers make it seem as though the focus of learning hematology is primarily learning the technology of a complete blood count (CBC). Although these analyzers do provide quick, reliable, and accurate results, it is still important for practitioners of laboratory medicine to recognize the need for manual review. It is vital for medical laboratory scientists (MLSs) to remain competent in the determination of cell morphology, including red blood cells, platelets, and white blood cells.

This chapter begins by first defining and illustrating normal blood cell morphology. Abnormal morphology is then discussed in terms of basic assessment techniques, with particular emphasis on recognizing distinct morphology and relating it to possible clinical conditions. Physiological mechanisms are explained to give the reader a better understanding of abnormalities and how they relate to different disease states. A careful and thorough examination using light microscopy in the optimal area on a well-made, well-stained peripheral smear provides an experienced observer with valuable information about morphology, whether normal or abnormal.¹

Automation in the Hematology Laboratory

Advances in hematology analyzers have significantly enhanced the ability of automation to perform and report a CBC analysis, from basic direct current impedance enumeration of cells to complex instruments that incorporate several different technologies in analyzers. Details on hematology automation are discussed later in Chapter 32: Automated Differential Analysis. However, it is important to understand when and why the review of a blood smear is indicated.

Although analyzers have the capability to detect abnormalities when these results are **flagged**, an operator must understand that the results require critical evaluation before the results can be released to the ordering doctor. In most cases, the instrument will specify the result with an indicator (flag) such as a symbol (i.e., an asterisk) or a high (H) or low (L) designation depending on the abnormality. The required review may include a **delta check**, that is, current patient results are compared with prior history or match a parameter that automatically requests further testing. This is referred to as **reflex testing**. Reflex testing of an abnormal or flagged result can include a slide review of abnormal morphology (usually scanning 8 to 10 oil immersion fields) and, if necessary, a complete white blood cell differential count. Investigating a delta check should include correlation with the patient's clinical presentation. However, it can indicate sample integrity errors such as platelet clumping and sample mix-ups. Required manual morphology reviews are determined by the parameters set through individual laboratory policies based on financial and regulatory standards as well as medical considerations.

With the development of sophisticated automated blood-cell analyzers, the number of CBC samples that require a blood smear has steadily diminished. In many clinical settings

it is 10% to 25%, depending on the facility patient population.² Even so, the blood smear remains a crucial diagnostic aid. Maintaining medical laboratory scientist competency in the identification of cellular abnormalities must be a high priority in the laboratory.

CRITICAL THINKING QUESTION

4-1 With the advancements in automated instrumentation, why is it necessary for medical laboratory science professionals to still have knowledge and skills pertaining to performing peripheral blood smear review?

See answers to all Critical Thinking Questions at the back of this book.

Examination of the Peripheral Blood Smear

All laboratories should have a documented protocol for initiating a blood smear examination along with standardized reporting guidelines. Considering that most of the blood smears that require review are abnormal, it is essential that students be able to first recognize normal blood cell morphology. From there, they should be able to determine whether a cell is abnormal, classify it, and relate it to a disease state.

Morphological abnormalities can often only be determined by a blood smear. An analyzer can flag a result, but only a competent morphologist can identify many cellular abnormalities. The microscopic examination of a peripheral blood smear provides a wealth of information to the clinician. It is used to detect or verify abnormalities and subsequently may provide the clinician with information from which a differential diagnosis can be made. Various forms of anemia may actually be diagnosed from abnormal red cell morphology reported on a blood smear examination. The report of abnormal white cell morphology may indicate what additional testing may be required. Abnormal platelet morphology may detect a platelet function deficiency even when normal numbers of platelets have been reported from the analyzer.

The examination of the blood smear should include evaluation of the red cell, white cell, and platelet morphology. The technologist should review at least 8 to 10 oil immersion fields (OIF) to thoroughly evaluate the smear. The red cell morphology evaluation should include examination for deviations in size, shape, distribution, concentration of hemoglobin, color, and the appearance of inclusions. The white cell morphology evaluation should consist of differentiation of the white blood cells and their overall appearance including nuclear abnormalities, cytoplasmic abnormalities, and the presence of abnormal inclusions that may denote a disease process. Platelet counts should be verified, and in addition, the smear should be reviewed for platelet shape and size abnormalities, and for clumping.

When abnormal morphology is identified on the smear, the scientist must determine whether the abnormality is possibly artifactual and not pathological. For example, refractile artifacts may be the result of water or stain contamination and should not be confused with red cell inclusions. Echinocytes

or crenated cells may also be artifacts if practically every cell in the thin portion of the film has a uniformly spiculed membrane.

The following describes the necessary steps in the examination of the stained peripheral blood smear.

Low-Power (10×) Scan

1. Determine the overall staining quality of the blood smear.
2. Determine whether there is a good distribution of the cells on the smear.
 - Scan the edges and center of the slide to be sure there are no clumps of RBCs, WBCs, or platelets.
 - Scan the edges for abnormal cells.
3. Find an optimal area for the detailed examination and enumeration of cells.
 - The RBCs should not quite touch each other.
 - There should not be areas containing large amounts of broken cells or precipitated stain.
 - The RBCs should have a graduated central pallor.

High-Power (40×) Scan

1. Determine the WBC estimate.
 - The WBC estimate is performed under high power (40× magnification). WBCs are counted in 10 fields and averaged. The estimate is reported according to the values given in Table 4-1.
 - The WBC estimate can also be performed using a factor based on the fact that each WBC seen in 400× magnification (high-power field) is equivalent to approximately 2,000 cells per μL of blood. For example, if the average number of WBCs counted per high power field was 5, the WBC estimate would be $5 \times 2,000$ or 10,000/ μL .
2. Correlate the WBC estimate with the WBC counts per mm^3 from the automated instruments.
3. Evaluate the morphology of the WBCs and record any abnormalities, such as toxic granulation, inclusions, or Döhle bodies.

Oil Immersion (100×) Examination

1. Perform a 100 WBC differential count.
 - Counting should be performed by moving in a zig-zag manner on the smear (Fig. 4-1).
 - All WBCs are to be included until a total of 100 have been counted.
2. Evaluate the RBCs for anisocytosis, poikilocytosis, hypochromasia, polychromasia, and inclusions.

3. Perform a platelet estimate and evaluate platelet morphology.

- Count the number of platelets in 10 OIFs.
- Divide by 10.
- Multiply by 15,000/ mm^3 if the slide was prepared by an automatic slide spinner; multiply by 20,000/ mm^3 for all other blood smear preparations.

4. Correct any total WBC count per mm^3 that has greater than 10 nucleated red blood cells (NRBCs) per 100 WBCs.

- When performing the WBC differential, do not include NRBCs in your count, but report them as the number of NRBCs per 100 WBCs.

- Use the following formula to correct a WBC count:

$$\text{CORRECTED WBCs/mm}^3 = \frac{\text{WBC/mm}^3 \times 100}{100 + \text{No. of NRBCs/100 WBCs}}$$

The examination of the peripheral blood smear is performed as part of the hematologic laboratory workup called the CBC.

CRITICAL THINKING QUESTION

- 4-2 Why is it important that the evaluation of WBC, RBC, and platelet morphology be conducted under oil immersion (100×), while broader evaluations may be performed under high-power (40×) magnification?

The Normal Red Blood Cell

To identify abnormal morphology, one must be competent in normal morphology identification and, more importantly, capable of differentiating them from abnormal cells. Therefore, we begin this section with a description of normal red blood cell (RBC) morphology. The mature erythrocyte (RBC, normocyte, discocyte) has a remarkable structure in that it lacks a nucleus and organelles, yet it has all components necessary for survival and function. It is described as a biconcave disc with a survival time of approximately 120 days in circulation. On a Romanowsky (i.e., Wright's, Giemsa)-stained blood smear, this mature red cell has a reddish-orange appearance. The RBC has an average diameter of 7 to 8 μm and an average volume of 90 fL. The area of central pallor is approximately 2 to 3 μm in diameter (Fig. 4-2), and the size variation of red cells from a normal patient is approximately 5%. The primary function of the red cell is the transportation of oxygen

TABLE 4-1 Estimation of Total WBC Count From the Peripheral Blood Smear

No./High-Power Field	Estimated Total WBC Count/ mm^3
2-4	4000-7000
4-6	7000-10,000
6-10	10,000-13,000
10-20	13,000-18,000



FIGURE 4-1 Blood smear made by the slide-to-slide (wedge) method. Counting should be performed by moving in a zig-zag manner on the smear to avoid counting the same cells.



FIGURE 4-2 Normal red blood cells.

to the tissues of the body and transportation of carbon dioxide back to the lungs for expulsion (see Chapter 2: The Red Blood Cell: Structure and Function).

Assessment of Red Cell Abnormality

A well-stained and well-made blood smear with an even distribution of RBCs in the area to be examined is essential for any peripheral blood smear review. If these criteria are achieved, the reviewer must make a general assessment of whether the morphological abnormality is due to shape change (**poikilocytosis**), size change (**anisocytosis**), or a change in color. Most assessments of anisocytosis are performed in concert with the red cell indices and the red cell distribution width (RDW) rating obtained from the hematology analyzer. In assessing the smear, the reviewer considers the percentage of cells that vary in size in at least 10 OIFs. For example, if the mean corpuscular volume (MCV) was 65 fL (80 fL to 100 fL normal range for adults), the reviewer would expect to see a large percentage of small cells. If the MCV were 105 fL, the reviewer would expect to see primarily larger cells. More information on red cell indices, including calculations, are presented in Chapter 6: Anemia: Diagnosis and Clinical Considerations and Chapter 31: Basic Hematology Procedures and Methods.

The majority of laboratories use either qualitative remarks (few or marked) or a numerical grading (1+ to 4+) based on the percentage of variation to describe the type of cell or cells that have caused the variation from the normal. With this method, a reviewer can present to the clinician a series of objective ratings that can translate to a visual impression of a patient's peripheral smear. This assessment may be critical to the provider's differential diagnosis of certain forms of anemia. Reviewers are urged to avoid the use of terms that are vague (e.g., the term "present") owing to the wide variations in the implication it may have to clinicians. See Table 4-2

TABLE 4-2 Grading Scale for Red Cell Morphology (Anisocytosis/Poikilocytosis)

Grade	Percentage of Cells That Differ in Size or Shape From Normal RBCs
Normal	5%
Slight	5%–10%
1+	10%–25%
2+	25%–50%
3+	50%–75%
4+	>75%
Sample Situations	
2+ Microcytes	Few schistocytes
1+ Macrocytes	Few burr cells
	1+ Target cells
3+ Anisocytosis	2+ Poikilocytosis

for an example of guidelines in grading anisocytosis and poikilocytosis. Please note that the assessment of RBC morphological abnormalities remains a manual task that is inherently subjective. It is imperative that laboratories establish guidelines based on their own patient and physician population. It is essential to patient care that the laboratory and clinician have similar interpretations of the results reported for all RBC morphology. Figure 4-3 is a composite chart of normal and abnormal red cell morphology.

Included in this chapter are flowcharts that correlate the abnormal morphology with a possible pathology. This scheme should enable the learner to easily associate an abnormal morphology with the clinical condition.

Variations in Red Cell Distribution

Distribution refers to how cells are dispersed on the peripheral smear slide. It is vital that red cells are distributed well to verify that the peripheral smear was made adequately and estimates of grading are accurate.

Normal Distribution

The area of the blood smear that is reviewed for morphological abnormalities is extremely important. The area to be reviewed should be in the area of the smear where the red cells are slightly separated from one another or, at most, barely touching with no overlap. This area should represent at least one-third of the entire film.³ The reviewer should avoid the thicker portion of the slide where cells are overlapping and the edges of smear where cells may be artifactually distorted in size, shape, and color. An exception is made when scanning for platelet clumping.

Abnormal Distribution

The abnormal distribution of red blood cells refers to areas where red cells are overlapping or stacked upon one another. These findings can signify inadequate slide-making processes.





























RED BLOOD CELL MORPHOLOGY					
Size variation	Hemoglobin distribution	Shape variation		Inclusions	Red cell distribution
Normal 	Hypochromia 1+ 	Target cell 	Acanthocyte 	Pappenheimer bodies (siderotic granules) 	Agglutination 
Microcyte 	2+ 	Spherocyte 	Helmet cell (fragmented cell) 	Cabot's ring 	Rouleaux 
Macrocyte 	3+ 	Ovalocyte 	Schistocyte (fragmented cell) 	Basophilic stippling (coarse) 	
Oval macrocyte 	4+ 	Stomatocyte 	Tear drop 	Howell-Jolly 	Crystal formation  
Hypochromic macrocyte 	Polychromasia (Reticulocyte) 	Sickle cell 	Burr cell 		

FIGURE 4-3 Normal and abnormal red blood cell morphology.

or be categorized as agglutination or rouleaux, which may aid in the differential diagnosis of certain pathological conditions.

Agglutination

Agglutination is an aggregation of red cells into random clusters or masses. Agglutination is the result of an antigen-antibody reaction within the body. In cases of autoagglutination, the reaction is actually with the patient's own cells and the patient's serum or plasma. Such is the case with cold antibody syndromes, for example, **cold hemagglutination** disease and **paroxysmal cold hemoglobinuria** (PCH) (see Chapter 14: Hemolytic Anemias: Extracorporeal Defects). Agglutination due to the previously mentioned disorders occurs at room temperature during sample preparation and appears as interspersed areas of clumping throughout the peripheral smear (Fig. 4-4). The use of saline will not disperse these agglutinated areas; however, warming the sample to 37°C helps to break up the agglutinins, allowing for the possibility of normal slide preparation for morphology review. The MCHC and MCV from these specimens are usually falsely elevated in response to the agglutinin formation. Other forms of autoagglutination may also occur spontaneously but are more likely to be seen in connection with certain hemolytic anemias, atypical pneumonia, staphylococcal infections, and trypanosomiasis. Agglutination is not to be confused with **rouleaux**.

Rouleaux

Rouleaux is a condition in which red cells appear as stacks of coins on the peripheral smear. The stacks may be short or long, but regardless of the length, the red cells appear stacked on one another. These stacks are rather evenly dispersed throughout the smear. Rouleaux formation is the result of elevated globulins or fibrinogen in the plasma, where the

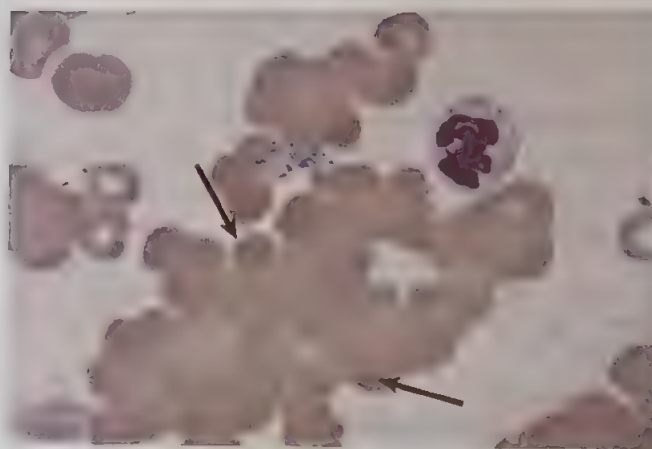


FIGURE 4-4 Note the agglutination on the smear from a patient with cold hemagglutinin disease.

red cells have been more or less "bathed" in this abnormal plasma, which gives them a sticky consistency. This lowers the zeta (ζ) potential, thus facilitating the stacking effect (Fig. 4-5). The use of a saline dilution of the serum disperses rouleaux. Rouleaux formation correlates well with a high erythrocyte sedimentation rate.

Rouleaux is seen in patients with **hyperproteinemias** such as multiple myeloma and Waldenström's macroglobulinemia (refer to Chapter 23). It may also be seen in chronic inflammatory disorders and some lymphomas (refer to Chapter 22). It is important to note that in cases of severe rouleaux it may be impossible to evaluate cell size or shape.

Peripheral smears reviewed in the thick portions of the smear, and entire smears made too thick, may appear to

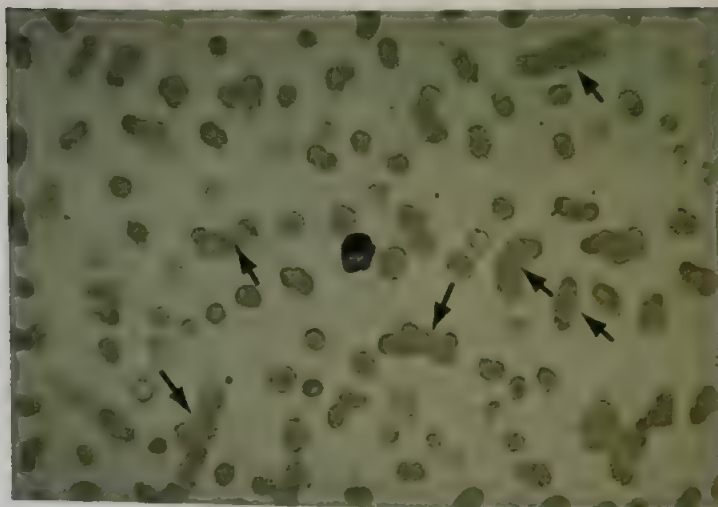


FIGURE 4-5 Peripheral blood showing marked rouleaux formation. Note the "stacked coin" appearance of the red cells.

exhibit rouleaux. This is considered artifactual and should not be reported until it is verified in the thin portion of the smear or a new slide is prepared.

CRITICAL THINKING QUESTION

- 4-3 How can one determine whether a finding of agglutination or rouleaux is artifactual or clinically significant?

Variations in Red Cell Size

Variations in the size of red blood cells (anisocytosis) should be evaluated when performing a peripheral smear review. Red cells may be found to be present in a variety of sizes, too large, or too small. Red cell sizes should be categorized as normocytes, macrocytes, or microcytes and graded appropriately. This section will review the designations for red cell anisocytosis.

Anisocytosis

Any significant variation in size is known as **anisocytosis** (Fig. 4-6). This size variation is frequently found in the leukemias and in most forms of anemia. The severity of the variation should also correspond to an increased red cell distribution (RDW). Anisocytosis results from abnormal cell development and typically results from a deficiency in the raw materials (i.e., iron, vitamin B₁₂, folic acid) needed to manufacture RBCs or by a congenital defect in the cell's structure. Cell size may deviate, from measuring smaller

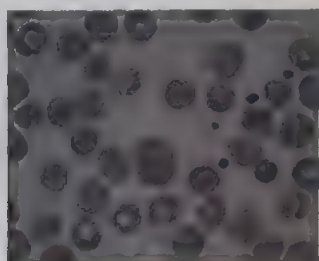


FIGURE 4-6 Note the variation in size (anisocytosis) of the red blood cells.

than the normal 7 μm , to being larger than normal. The terms used to describe these abnormalities are **microcyte** ($\leq 6 \mu\text{m}$) and **macrocyte** ($\geq 9 \mu\text{m}$). These terms are used in conjunction with the terms "microcytosis" and "macrocytosis" and should also correlate with the red cell indices results. Anisocytosis is graded in most facilities as 1+ to 4+ (see Table 4-2). When reporting anisocytosis, it is important to describe the morphology picture in terms of microcytosis or macrocytosis, or in cases of a dimorphic population, there may be the appearance of both.

Normocytes

The average size of the erythrocyte is indicated by the measurement of the MCV, a result generated by the automated hematology analyzer. The MCV is considered an integral part of a CBC. Observation of red cell morphology on the blood smear provides a quality control check on the electronic MCV, as well as the other two red cell indices, mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC).⁴ A "normal" MCV would correspond to the MCV reference range (80 to 100 fL for adults). Subsequent review of the blood smear should yield no significant size variation from the normal 7- to 8- μm red cell. This scenario is referred to as **normocytic**, and the red cells are classified as **normocytes**. This information would prove useful to the physician in the diagnosis of anemia. In the case of a normal MCV and a high RDW (normal RDW is 11.5% to 14.5%), the reviewer would expect to see a mixture of large and small cells. This scenario is referred to as a **dimorphic population** and may be the result of a recent blood transfusion or possibly the patient may be in the recovery stages of anemia. Patient history plays an important role in this situation.²

Macrocytes

Macrocytes are red blood cells that are approximately 9 μm or larger in diameter, having an MCV of greater than 100 fL. Anemias associated with these cells are referred to as **macrocytic** and are classified as either megaloblastic or nonmegaloblastic.⁵ These large red cells may appear in the peripheral circulation by several mechanisms. One mechanism is impaired deoxyribonucleic acid (DNA) synthesis which results in **megaloblastic** erythropoiesis leading to fewer cellular divisions and consequently a larger cell. This form of erythropoiesis produces a **megaloblastic anemia** and may be the result of B₁₂ or folate deficiency, chemotherapy, or any process producing a nuclear maturation defect. Macrocytes with an oval shape (**macroovalocytes**), neutrophilic hypersegmentation, as well as MCV values exceeding 120 fL are typically seen in this type of anemia (refer Chapter 8).

The most common cause of **nonmegaloblastic** macrocytosis is accelerated erythropoiesis, which results from conditions such as acute blood loss or alcoholism. The cells are released prematurely from the marrow, are nonnucleated, and appear larger than a mature erythrocyte. On a Wright-stained smear the cells will appear as round polychromatophilic macrocytes and on a supravital (i.e., new methylene blue)-stained smear they appear as reticulocytes. Neutrophilic hypersegmentation is not typically seen in this form of macrocytosis.³

Macrocytosis may result from other conditions, such as hypothyroidism and various bone marrow disorders, as well as occur in neonatal blood and postsplenectomy. Additionally, in cases where excess plasma cholesterol may be taken up by the red cell, it subsequently leads to an increase in the surface area of the cell. However, this last mechanism may not be reflective of a "true" macrocytosis (obstructive liver disease). Macrocytes should be evaluated for shape (oval versus round), color (red versus blue), pallor (if present), and the presence or absence of inclusions. The conditions in which macrocytes may be seen are listed in Figure 4-7.

Microcytes

A **microcyte** is a small red blood cell having a diameter of less than 7 μm and an MCV of less than 80 fL. Anemias associated with microcytes are said to be **microcytic**. The hemoglobin content of these cells may be normal to decreased. A consequence of any defect that results in impaired hemoglobin synthesis may produce a microcytic, hypochromic (MCHC $<32\%$ and cells with increased central pallor) blood picture. When erythroid cells are deprived of any of the essential elements in hemoglobin synthesis, the result is an increase in cellular divisions and consequently a smaller cell in the peripheral blood. This form of abnormal hemoglobin synthesis is seen in iron deficiency, deficiency of heme synthesis (sideroblastic anemia), deficiency of globin synthesis (thalassemia), and chronic disease states. In

the case of iron deficiency, microcytosis will not be visually apparent until iron stores in the body have been completely exhausted and iron deficient erythropoiesis takes place, as in **iron deficiency anemia (IDA)** (see Chapter 7).

ADVANCED CONTENT

Decreased or defective globin synthesis also presents as a microcytic/hypochromic anemia, but in most cases, this results from a genetic abnormality producing a hereditary anemia known as **thalassemia** (see Chapter 12). This microcytic/hypochromic anemia is rare, and in the homozygous form, it may result in a severe anemia with a high rate of mortality. In the milder heterozygous form, this anemic picture may be confused with IDA. The appearance of target cells, family studies, as well as additional hematological testing may be needed for a differential diagnosis.

It is important to note that other disease processes such as sideroblastic anemia and lead poisoning may produce significant numbers of microcytic red cells, in most cases without hypochromia.

Clinical conditions in which microcytes may be seen as the predominant cell morphology are illustrated in Figure 4-8.

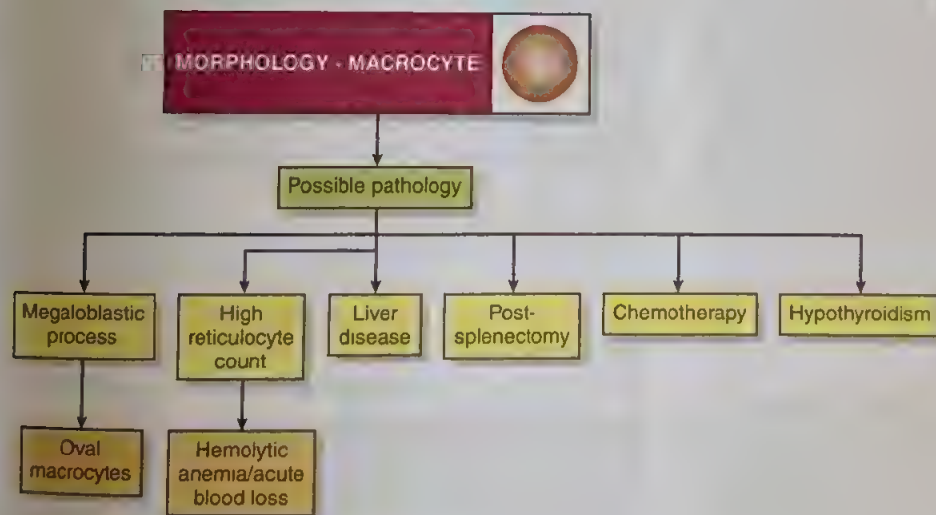


FIGURE 4-7 Correlation of macrocytes to pathological processes.

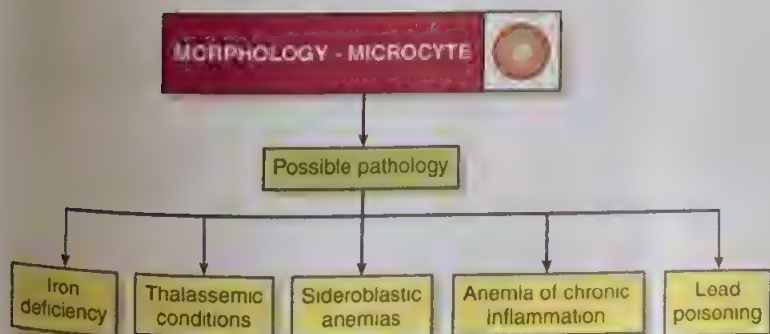


FIGURE 4-8 Correlation of microcytes to pathological processes.

Hemoglobin Content—Red Cell Color Variations

Color variations of hemoglobin content include normochromia, hypochromia, hyperchromia, and polychromasia.

Normochromia

The term **normochromic** indicates the red cell is essentially normal in color. A normochromic erythrocyte has a well-hemoglobinized cytoplasm with a small but distinct zone of central pallor. The area of pallor does not exceed 3 μm when measured linearly. The term “normochromic” is used to describe an anemia with a normal MCHC and MCH. When used in conjunction with a normal MCV, the anemia would be described as a **normochromic/normocytic anemia** (see Fig. 4-2).

Hypochromia

Any RBC having a central area of pallor of greater than 3 μm is said to be **hypochromic**. There is a direct relationship between the amount of hemoglobin deposited in the red cell and the appearance of the red cell when properly stained. The term “hypochromia” literally means “low color” and indicates that the cells have less than the normal amount of hemoglobin. Typically, any irregularity in hemoglobin synthesis will lead to some degree of hypochromia (Fig. 4-9).

Most clinicians choose to assess hypochromia based on the mean corpuscular hemoglobin concentration (MCHC), which by definition measures hemoglobin content in a given volume of red cells (100 mL). When the MCHC is $<32\%$, the anemic process is described as being hypochromic, and the slide reviewer should scan the peripheral smear and expect to see RBCs with increased central pallor or hypochromia. A lower MCHC result typically correlates with a larger central pallor in the affected red cells. In general, this is very reliable; however, it does not consider the situation in which a true hypochromia is observed in the presence of a normal MCHC. In many cases, the MCHC will not be consistent with what is observed on the peripheral smear. The morphologist should not be unduly influenced by the RBC

indices in the evaluation of hypochromia. True hypochromia will appear as a delicate shaded area of pallor as opposed to **pseudohypochromia** (the water artifact), in which the area of pallor is distinctly outlined. It is important to note that not all hypochromic cells are microcytic. Target cells (discussed later in this chapter) possess some degree of hypochromia, and there are macrocytes and normocytes that can be distinctly hypochromic. Refer to Table 4-3 for a guideline to grading hypochromia.

CRITICAL THINKING QUESTION

4-4 Why would an anemia cause RBCs to be hypochromic?

The most common condition manifesting hypochromia is IDA, yet any reduction in the amount of stored iron can cause this type of anemia.⁶ In severe cases of IDA, red cells exhibit an inordinately thin band of hemoglobin. Patients with iron deficiency may have many hypochromic cells, depending on the magnitude of the deficiency. In addition to large numbers of hypochromic cells, there may be large numbers of microcytes as well. Iron deficiency anemia is commonly referred to as a microcytic/hypochromic anemia.

ADVANCED CONTENT

In the alpha (α) and beta (β) heterozygous (trait) thalassemia syndromes hypochromia is much less pronounced. However, red cells in the α -thalassemias and β -thalassemia homozygous states show significant amounts of pallor⁷ (see Chapter 12). Sideroblastic anemias show a prominent dimorphic blood picture—macrocytic, normocytic, and microcytic cells together, only some of which show true hypochromia. Some hypochromic cells may be seen in patients with lead poisoning.

Hyperchromia

Red cells with a decreased surface-to-volume ratio and a decreased or absent central pallor may be described as **hyperchromic**. True hyperchromia exists when the MCHC is $>36\%$ and may be seen in the peripheral smears of patients with hemolytic anemias, including hemolysis caused by burns

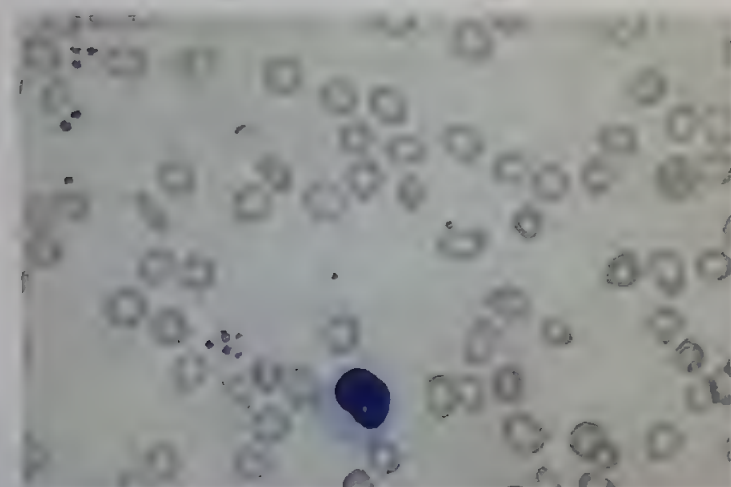


FIGURE 4-9 Note the large central pallor in many of the red cells depicting hypochromia.

TABLE 4-3 Hypochromia Grading

Grade	Description
1+	Area of central pallor is one-half of cell diameter
2+	Area of pallor is two-thirds of cell diameter
3+	Area of pallor is three-quarters
4+	Thin rim of hemoglobin

and other toxicities. Even though true hyperchromia does exist, it is not reported as such without verification with additional testing.⁷ It is reported in terms of the cell abnormalities resulting from the increased volume of hemoglobin and the decreased surface area. The cell produced from these phenomena appears as a solid reddish-orange disc with no central pallor and is referred to as a **spherocyte**, which is discussed later in this chapter.

Polychromasia

When RBCs are delivered to the peripheral circulation prematurely, their appearance in the Wright-stained smear is distinctive. These red cells are described as **polychromatophilic** (diffusely basophilic) and are gray-blue in color and usually larger than normal red cells (Fig. 4-10). The basophilic color of the red cell is the result of the residual RNA involved in hemoglobin synthesis. Polychromatophilic macrocytes, as seen on a Wright's-stained smear, are actually reticulocytes; however, the reticulum cannot be visualized without supravital staining.

Because regeneration of red cells is a dynamic process, it is not uncommon to find a few polychromatophilic cells in a normal peripheral blood smear. The reticulocyte count should reflect the degree of polychromasia. Polychromatophilic red cells appear in varying shades of blue on the stained blood smear. Any clinical condition in which the marrow is stimulated, particularly RBC regeneration, will produce a polychromatophilic blood picture. This represents effective erythropoiesis as well as an assessment of bone marrow function. Examples of several conditions in which polychromasia is noted include acute and chronic hemorrhage, hemolysis, and any regenerative red cell process. The degree of polychromasia is an excellent indicator of therapeutic effectiveness when a patient is given iron or vitamin therapy as a treatment for anemia. Refer to Table 4-4 for a guideline to polychromasia grading.

Variations in Red Cell Shape

Variations in red cell shape (poikilocytosis) include the following: target cells (codocytes), spherocytes, stomatocytes,

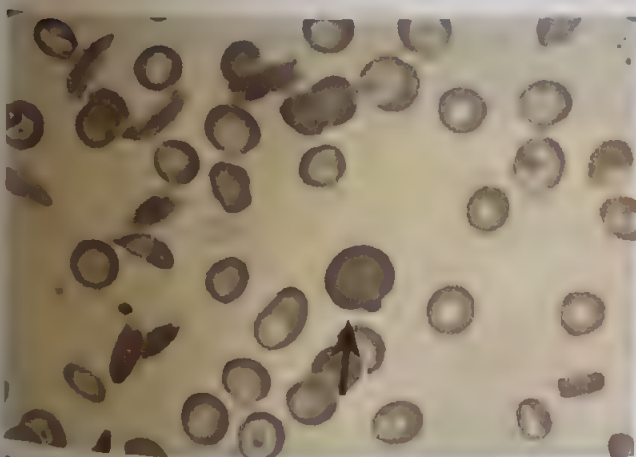


FIGURE 4-10 Note polychromasia in the cell with the arrow.

TABLE 4-4 Polychromasia Grading

Grade	Percentage of Red Cells That Are Polychromatophilic
Slight	1%
1+	3%
2+	5%
3+	10%
4+	>11%

ovalocytes and elliptocytes, sickle cells (drepanocytes), fragmented cells (schistocytes, helmet cells, keratocytes), burr cells (echinocytes), acanthocytes (thorn cells, spur cells), and teardrop cells (dacrocytes).

Poikilocytosis

Poikilocytosis is the term used to describe a variation in red cell shape (Fig. 4-11). Normal erythrocytes vary only slightly from the concise round shape of a biconcave disc, so even a slight variation in significant numbers may prove to be important. These **poikilocytic** cells may take on such peculiar shapes as teardrops, pencils, and sickles. The differential diagnosis of anemia cannot be determined from a reported poikilocytosis. The term should be used in conjunction with more descriptive terminology that would specify the particular morphological abnormality observed. Examples of specific poikilocytes are sickle cells, which result from abnormal hemoglobin, and spherocytes, which result from a red cell membrane abnormality as many of the poikilocytic cells do. The differential diagnosis of some forms of anemia may be determined by identification of a specific morphological abnormality.

The term "poikilocytosis" refers to the entire red cell morphology in the scanned area of a peripheral smear and is graded as 1+ to 4+ (see Table 4-2). Many labs consider the term poikilocytosis as a "catch-all" phrase for abnormal red cells and in lieu of grading the smear for poikilocytosis opt only to grade the specific types of morphologically abnormal cells seen. In these cases, the particular cells should be reported in terms of few, moderate, and many.

Target Cells (Codocytes)

Target cells appear on the peripheral blood because of an increase in RBC surface membrane. They are artificially



FIGURE 4-11 Note the extreme variation in RBC shape (poikilocytosis).

induced on the smear, and their true circulating form, as seen with an electron microscope, is a bell-shaped cell. The name **codocyte** is from the Greek word "kodon," meaning bell. In air-dried smears, however, they appear as "targets," with a large portion of hemoglobin displayed at the rim of the cell and a portion of hemoglobin that is central, eccentric, or banded (Fig. 4-12). As the name implies, the cell actually resembles a target and is sometimes referred to as a "bull's eye" cell. Target cells are seen in many types of anemia; however, they are most prominent in the hemoglobinopathies, thalassemias, and liver disease, and are also found postsplenectomy.² Correlation of target cells to pathological processes is shown in Fig. 4-13.

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The mechanism of targeting is related to excess membrane cholesterol and phospholipid and decreased cellular hemoglobin. This is well documented in patients with liver disease, in whom the cholesterol/phospholipid ratio is altered and can also be found in familial lecithin-cholesterol acyltransferase deficiency (LCAT).² Mature

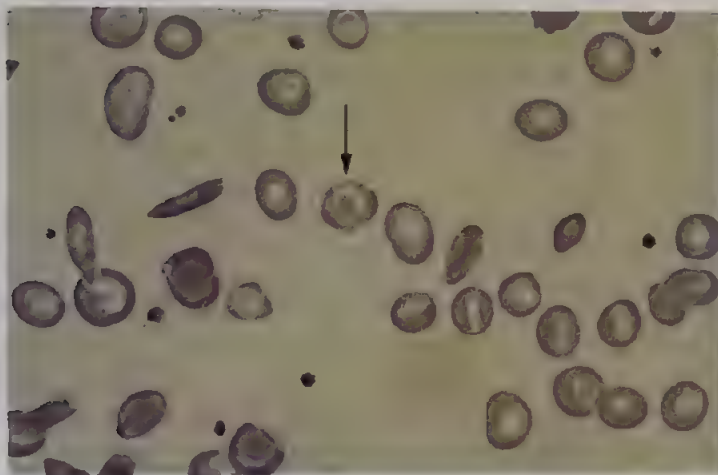


FIGURE 4-12 Note the target cell at the arrow.

red cells are unable to synthesize cholesterol and phospholipid independently. As cholesterol accumulates in the plasma, as seen in liver dysfunction, the red cell is expanded by increased membrane lipid, resulting in increased surface area. Consequently, the osmotic fragility is also decreased (see Chapter 31). The target cells are reversible in the case of LCAT.²

Spherocytes

Spherocytes have a reduced surface-to-volume ratio that results in a cell with no central pallor. Because of their density (intense color) and smaller size, they are easily distinguished in a peripheral smear. Their shape change is irreversible and may also be seen as **microspherocytes**. They are considered the most common form of the erythrocyte morphological disorders stemming from an abnormality of the cell membrane. This abnormality may be hereditary or acquired and may be produced by a variety of mechanisms affecting the red cell membrane. Figure 4-14 lists the more common pathological conditions in which spherocytes are seen. Perhaps the most detailed mechanism for spherizing is the congenital condition known as **hereditary spherocytosis** (HS) (see Chapter 9). This is an inherited, autosomal dominant condition and is due to a deficiency of, or a dysfunction in, the membrane proteins spectrin, ankyrin band 3, and/or protein 4.2.⁸ Figure 4-15 depicts a blood smear from a patient with hereditary spherocytosis.

ADVANCED CONTENT

Hereditary spherocytosis is the most common inherited hemolytic anemia. The membrane cytoskeleton of the RBC is dependent on particular proteins previously listed to maintain the shape, deformability, and elasticity of the red cell. The deficiency and/or dysfunction of any one of these membrane components will destabilize the cytoskeleton, resulting in abnormal red cell morphology.

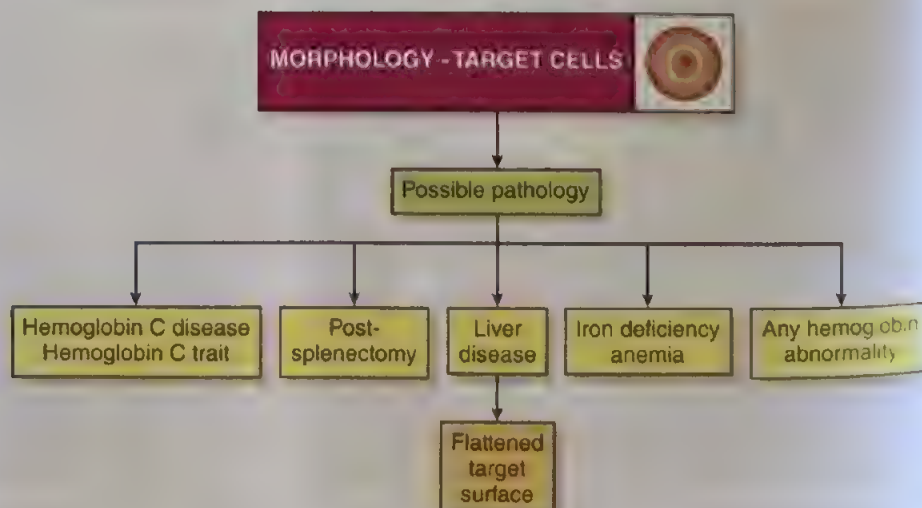


FIGURE 4-13 Correlation of target cells to pathological processes.

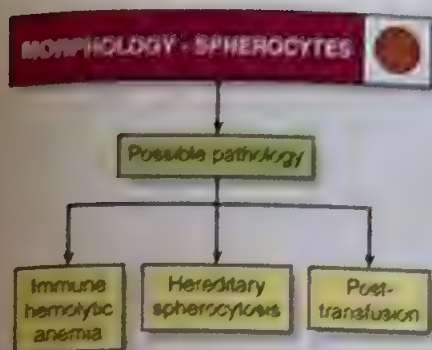


FIGURE 4-14 Correlation of spherocytes to pathological processes.

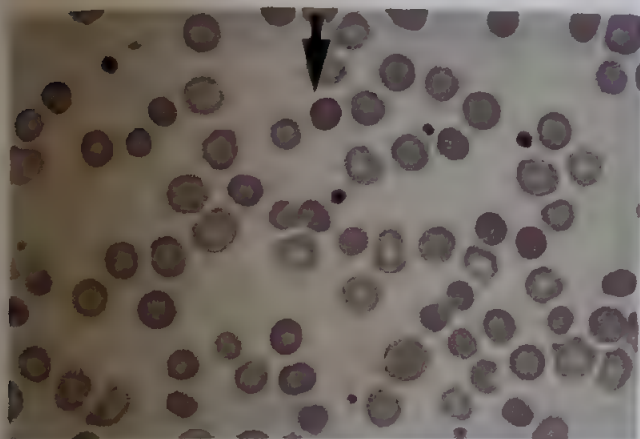


FIGURE 4-15 Note the spherocyte at arrow in a blood smear from a patient with hereditary spherocytosis.

and a shorter life span for the affected red cells in circulation.⁸ Spherocytes are typically seen in large numbers in peripheral smears from patients with HS. Premature destruction of these abnormal erythrocytes in the spleen may produce a mild to severe hemolytic anemia depending on the severity of the abnormality. Erythrocytes from patients with hereditary spherocytosis have a mean influx of sodium twice that of normal cells. Because these spherocytes have increased ability to metabolize glucose, they can handle the excessive intracellular sodium while in the plasma, but when they reach the microenvironment of the spleen, the active passive transport system is unbalanced with increased sodium and decreased glucose resulting in swelling and hemolysis of these cells (see Chapter 9). Historically, an increase in the MCHC and RDW have been associated with the CBC results of a patient with HS. More recently, additional measurements and molecular testing have proven to be more useful in diagnosing HS.⁹

The acquired forms of spherocytosis share the mutual defect with HS in that there is a loss of membrane. In the normal aging process of red cells, they gradually lose their

functionality through loss of cellular lipids, proteins, etc.; thus, spherocytes are produced as a final stage before aged red cells are detained in the spleen and trapped by the reticuloendothelial system. This natural process does not typically result in anemia. Another mechanism of producing spherocytes that may result in a mild to severe anemia is autoimmune hemolytic anemia. The coating of the red cells with antibodies and the detrimental effect of complement activation results in the membrane loss of cholesterol accompanied by a loss of surface area without hemoglobin loss producing spherocytes. The reduced surface-to-volume ratio of all spherocytes renders them abnormally susceptible to osmotic lysis; consequently, they have an increased osmotic fragility. Hemolysis is known to result from membrane abnormalities; therefore, other hemolytic processes may also produce spherocytes. They may also be seen as microspherocytes in the peripheral smears of burn patients.

Stomatocytes

The word stomatocyte is derived from the Greek word "stoma," which means mouth. They have a central pallor, which is said to be slit-like or mouth-like on peripheral blood smears. These red cells are of normal size, but are not biconcave, and in wet preparations appear bowl-shaped (Fig. 4-16). The abnormal morphology resulting in the stomatocyte is thought to be the result of a membrane defect (see Chapter 9).

ADVANCED CONTENT

Stomatocytosis is associated with abnormalities in red cell cation permeability that lead to changes in red cell hydration and volume, which may be either increased (hydrocytosis) or decreased (xerocytosis), or in some cases, near normal.¹⁰ While hydrocytosis and xerocytosis represent the extremes of a spectrum of red cell permeability defects,



FIGURE 4-16 Stomatocytes in peripheral blood.

they are more commonly secondary to other disorders such as hemoglobinopathies.¹⁰ The exact physiological mechanism of stomatocytic shape is poorly understood, yet the molecular basis of this disorder has been recently shown to be associated with a mutation in the PIEZO1 protein.¹¹ Stomatocytosis may be acquired or congenital. As with hereditary spherocytosis, stomatocytes are seen in significant numbers in the hereditary form known as **hereditary stomatocytosis** and in smaller numbers in the acquired form. Many chemical agents can induce stomatocytosis in vitro (phenothiazine and chlorpromazine); however, these changes are reversible.¹²

Stomatocytes are known to have an increased permeability to sodium; consequently, their osmotic fragility is increased. Stomatocytes are more often artifactual than a true manifestation of a particular pathophysiologic process. The artifactual stomatocyte has a distinct slit-like area of central pallor, whereas the area of pallor in the genuine stomatocyte appears shaded. Hereditary stomatocytosis is usually a benign condition or at most a mild normochromic/normocytic anemia. Occasional stomatocytes might be found in hemolytic anemia, alcoholic cirrhosis, and acute alcoholism.

ADVANCED CONTENT

Stomatocytosis is also present on peripheral blood smears of patients with Rh deficiency syndrome, also known as Rh null disease, in which erythrocytes from these rare individuals have either absent (Rh_{null}) or markedly reduced (Rh_{mod}) Rh antigen expression. This may result in a mild to moderate hemolytic anemia, and mutations in the *Rh30* and *RhAG* genes have been associated with this syndrome.¹³

Ovalocytes and Elliptocytes

Many investigators consider the terms **ovalocyte** and **elliptocyte** to be interchangeable; however, for the purposes of this discussion, they are viewed as distinct and separate. This morphological abnormality is thought to be the result of a mechanical weakness or fragility of the membrane skeleton and may be acquired or congenital. The pathogenesis of the formation of either of these cells is unknown. **Ovalocytes** may be considered as more egg-shaped and have a greater tendency to vary in their hemoglobin content. They can appear normochromic or hypochromic, normocytic or macrocytic. Megaloblastic anemia is characterized by oval macrocytes (**macroovalocytes**) that may be 9 μm or more in diameter and lack central pallor (Fig. 4-17).¹⁴

Elliptocytes, on the other hand, are pencil-, rod-, or cigar-shaped, and hemoglobin appears to be concentrated on both ends of the cell. They are invariably not hypochromic, exhibiting a normal central pallor.

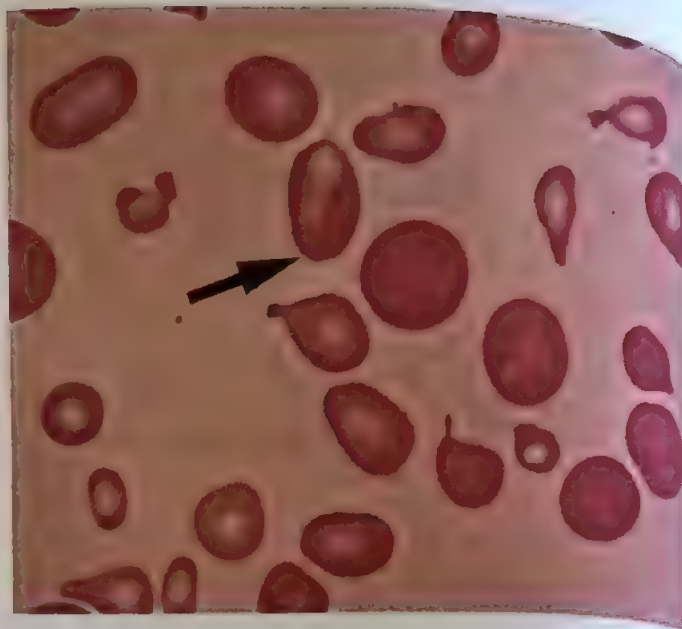


FIGURE 4-17 Note the oval macrocyte (macroovalocyte) at the arrow. Smear from a patient with pernicious anemia.

ADVANCED CONTENT

Hereditary elliptocytosis (HE) is a rare inherited condition with anywhere from 25% to 90% of all cells demonstrating the elliptical appearance. The erythrocytes in HE, in most cases, have a normal survival time; patients are typically asymptomatic and are diagnosed incidentally during testing for unrelated conditions.¹⁴ In approximately 10% of cases where red cell survival time is shortened, patients' symptoms may vary from a mild to severe transfusion-dependent hemolytic anemia. HE is caused by mutations in the red cell membrane protein α -spectrin, β -spectrin, or protein 4.1R (Fig. 4-18).¹⁵

Ovalocytes/elliptocyte may be seen in association with several disorders in addition to those already mentioned such as microcytic/hypochromic anemia, myelodysplastic syndromes, and myelophthistic anemia.²

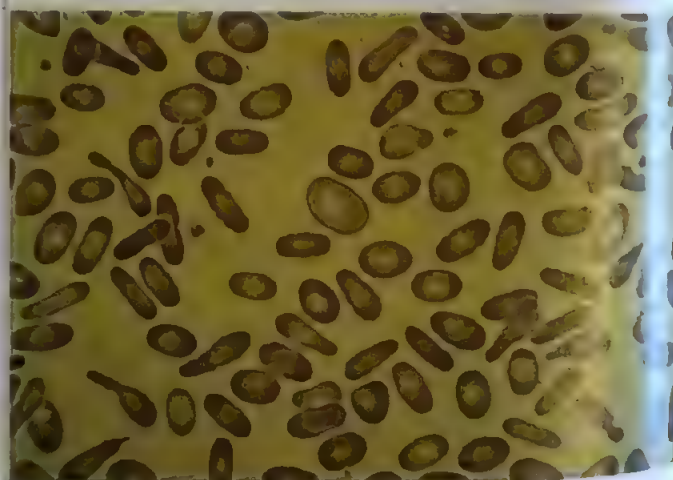


FIGURE 4-18 Note the high percentage of elliptocytes in this blood smear from a patient with hereditary elliptocytosis.

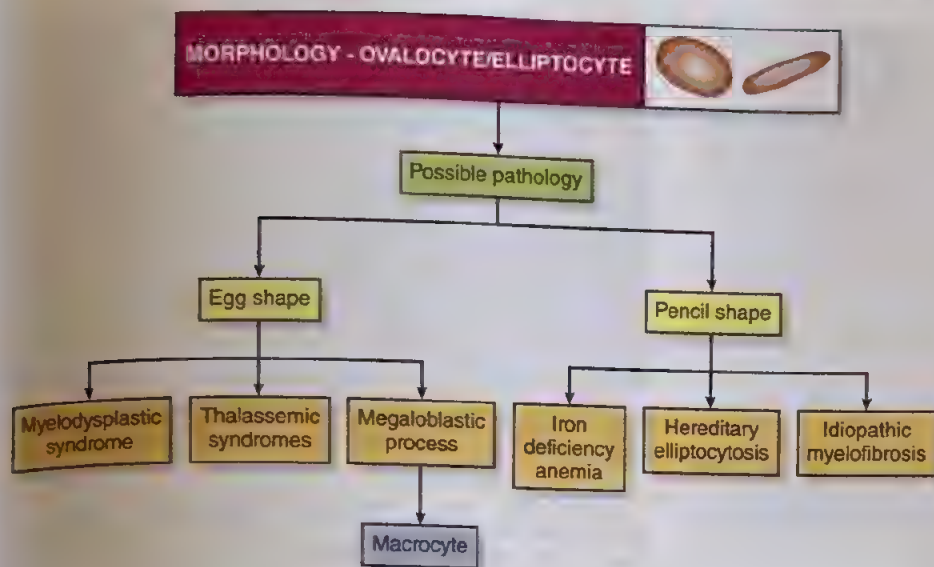


FIGURE 4-19 Correlation of ovalocytes and elliptocytes to pathological processes.

Refer to Figure 4-19 for a description of pathological processes associated with ovalocytes and elliptocytes (see Chapter 9).

Sickle Cells (Drepanocytes)

Drepanocytes or sickle cells are typically crescent- or sickle-shaped with pointed projections at one or both ends of the cell. These cells have been transformed by hemoglobin polymerization into rigid, inflexible cells no longer resembling the normal biconcave disc (Fig. 4-20). Patients may be homozygous or in some cases heterozygous for the presence of the abnormal hemoglobin, hemoglobin S. In the homozygous patient, physiological conditions of low oxygen tension (in vivo or in vitro) cause the abnormal hemoglobin to polymerize, forming tubules that line up in bundles to deform the cell. The surface area of the transformed cell is much greater, and the normal elasticity of the cell is severely restricted. These cells have lost their ability to deform and in many cases are unable to negotiate the microvasculature of the tissues, which leads to oxygen deprivation in those areas (see Chapter 11).

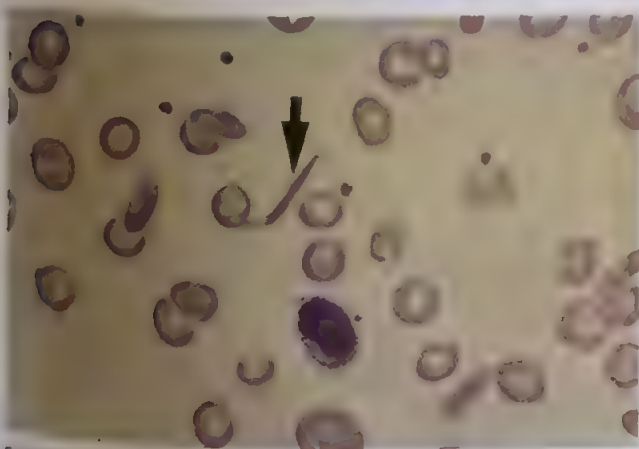


FIGURE 4-20 Irreversibly sickled cells

ADVANCED CONTENT

Most sickled cells possess the ability to revert to the discocyte shape when oxygenated; however, approximately 10% are incapable of reverting to their normal shape. These irreversibly sickled cells (ISCs) are the result of repeated sickling episodes. On the peripheral smear, they appear as crescent-shaped cells with long projections. When reoxygenated, the ISCs may undergo fragmentation. During a symptomatic period, the percentage of ISCs varies tremendously, and, consequently, it does not correlate with symptomatology. Sickle cells are not usually seen in the peripheral smears of individuals who are heterozygous (Hgb AS) and are only rarely seen in conjunction with other abnormal hemoglobins (i.e., Hgb C_{Harlem}, Hgb S_{Memphis}). Classically, sickled cells are best seen in wet preparations. Many of the cells observed on the Wright-Giemsa stain are the oat cell-shaped form of the sickled cell (Fig. 4-21). In this form, the projections are

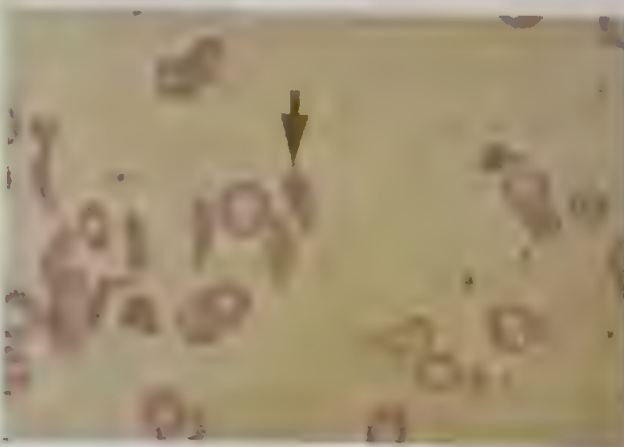


FIGURE 4-21 Reversible, oat-shaped sickle cell.

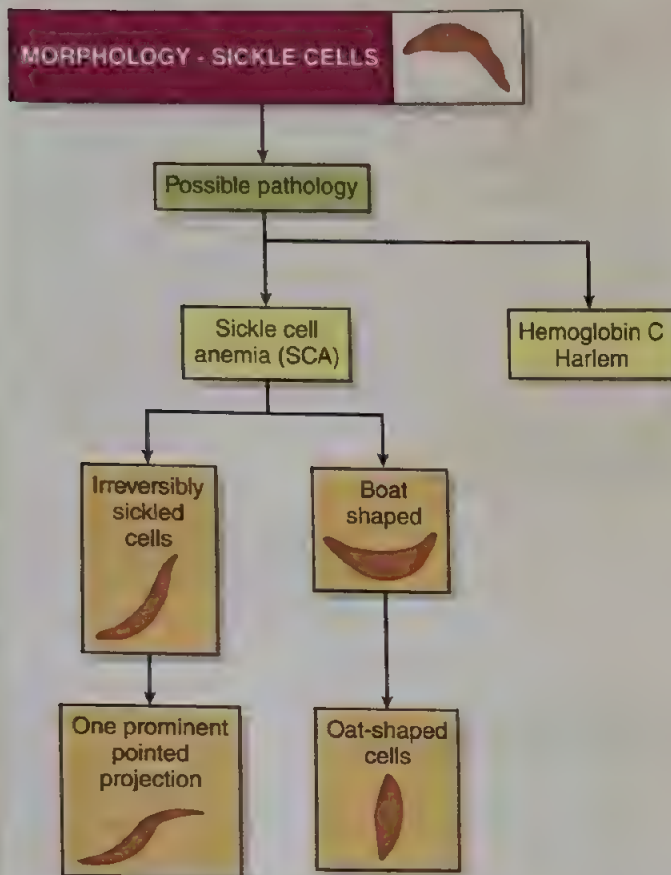


FIGURE 4-22 Correlation of sickle cells to pathological processes.

much less pronounced, and the central area of the cell is fairly broad. This shape is reversible.¹⁶ The more prominent pathological conditions in which sickle cells may be observed are listed in Figure 4-22. In this figure, a morphological distinction is made between ISCs and reversibly sickled cells.

Fragmented Cells

This section describes schistocytes, keratocytes, and helmet cells as fragmented cells. Please note that all laboratories may not report fragmented red cells in the same manner (i.e., all fragmented red cells reported as schistocytes) owing to the similarities in their origins. Regardless of the specificity of the terminology used, it is imperative that the morphologists give a qualitative estimate of the abnormality seen in all fields. Especially in significant numbers, the appearance of fragmented red cells will provide physicians with important information on the condition of their patients.

Refer to Figure 4-23 for a flowchart correlation of the fragmented cells matched to the pathological processes in which they may be observed.

Schistocytes

Schistocytes are split, cut, or cloven cells resulting from some form of trauma to the cell membrane. It is recognized that not all membrane alterations occur pathologically. However, there are certain triggering events in disease that invariably lead to fragmentation such as alteration of normal fluid circulation. Examples of fluid alterations are the development of fibrin strands, damaged endothelium, or a damaged heart valve prosthesis. The flow of blood in the circulation may actually sweep the erythrocytes through the fibrin strands, splitting the red cell. The shapes of these cells vary based on the shear forces and presentation of the red cells as they are cut by the fibrin. Intrinsic defects of the red cell make it less deformable and, therefore, more likely to be fragmented as it traverses the microvasculature of the spleen. Examples such as antibody-altered red cells and red cells containing inclusions have significant alterations that increase their likelihood of being fragmented, consequently decreasing their survival time.

Schistocytes are the extreme form of red cell fragmentation (Fig. 4-24). Whole pieces of red cell membrane appear to be missing, and bizarrely shaped red cells are apparent.

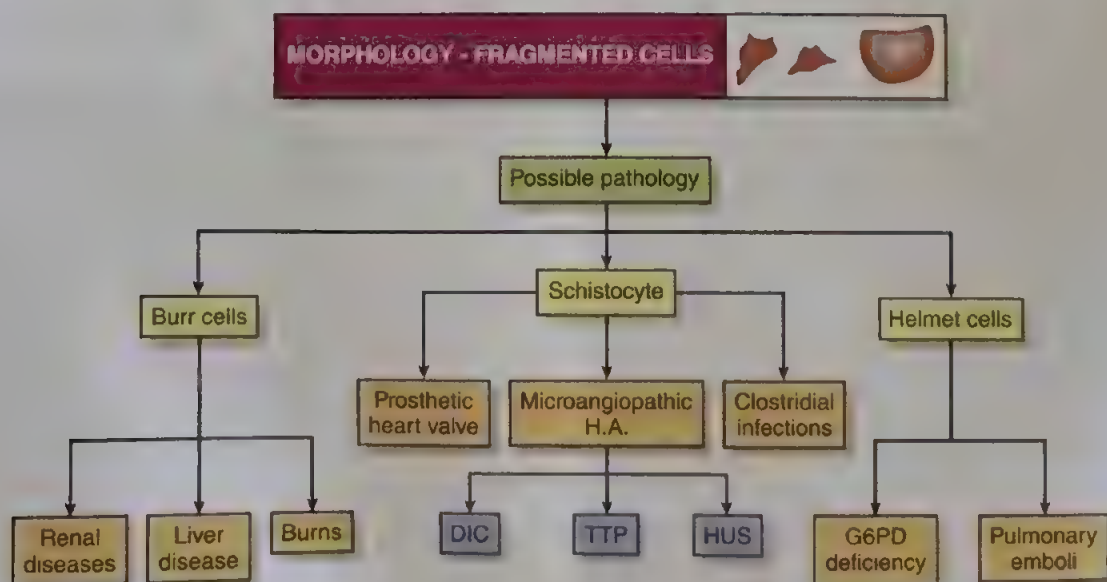


FIGURE 4-23 Correlation of fragmented cells to pathological processes. HA = hemolytic anemia; DIC = disseminated intravascular coagulation; HUS = hemolytic uremic syndrome; TTP = thrombotic thrombocytopenic purpura.

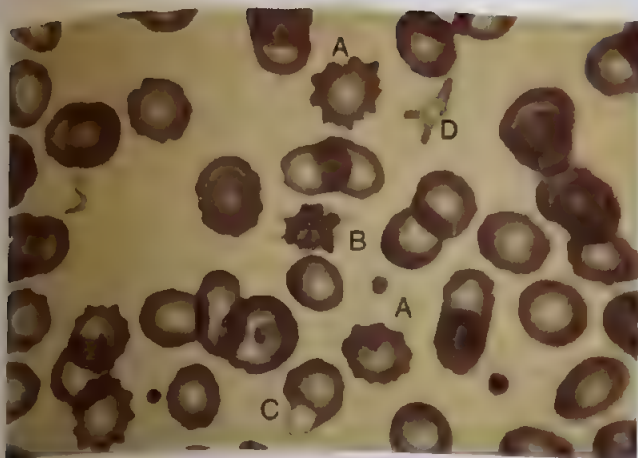


FIGURE 4-24 Peripheral blood from a patient with renal disease. Note the presence of fragmented cells: A. burr cells; B. acanthocyte; C. blister/pocketbook cells; D. schistocyte.

ADVANCED CONTENT

Schistocytes may occur in patients with thrombotic microangiopathic hemolytic anemia, disseminated intravascular coagulation (DIC), heart valve surgery, hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, HELLP (hemolysis, elevated liver enzymes, low platelet count) syndrome, malignant hypertension, severe burns, and hemoglobinopathies. While automated hematology analyzers are exploring the quantitation of RBC fragments, the manual morphological identification of schistocytes remains the gold standard in diagnosing severe and often fatal hemolytic disorders.¹⁵

Keratocytes

Keratocytes are red cells that have been caught on fibrin strands in circulation, and rather than splitting, the cell hangs over the fibrin fusing two sides of the cell together, creating a vacuole. Once the cell escapes from the fibrin strand, it appears in the peripheral blood as a red cell with a vacuole in one end resembling a blister and is called a **blister cell**. It also is said to resemble a women's handbag and may be called a **pocketbook cell** (Fig. 4-24). Once the vacuole ruptures, the resulting cell appears to have two horns. This "horned" cell also resembles a helmet and is sometimes reported as such but is actually a **keratocyte** (Greek for "keras," horn).¹⁷ The primary difference in the two cells is not in their appearance but in their formation.

Helmet Cells

The **helmet cell** also has distinctive projections, usually two, surrounding an empty area of the red cell membrane. Helmet cells are seen in hematological conditions in which large inclusion bodies are formed (Heinz bodies, Howell-Jolly bodies). Fragmentation occurs by the pitting mechanism of the spleen. This pitting mechanism removes the inclusion from the cell, giving the appearance of having taken a "bite out of the cell" and is sometimes referred to as a **bite cell** (Fig. 4-25). A helmet cell and a bite cell are, therefore, one and the same. The helmet cells may also be seen in patients with pulmonary emboli,

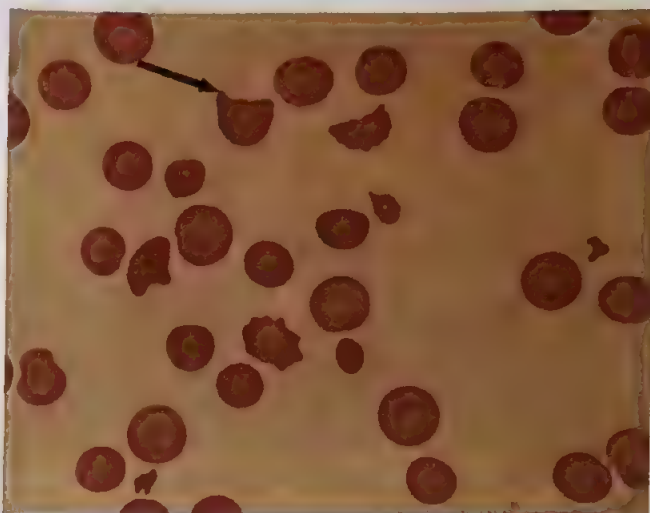


FIGURE 4-25 Note the bite or helmet cell at the arrow.

myeloid metaplasia, and DIC. All fragmented red cells are considered fragile, and their survival time is diminished significantly to days, if not hours, owing to splenic sequestration.

CRITICAL THINKING QUESTION

4-5 Why would a hemolytic process display schistocytes on the peripheral blood smear?

Burr Cells (Echinocytes)

Burr cells (echinocytes) are red cells with approximately 10 to 30 rounded spicules evenly placed over the surface of the red cells (refer to Fig. 4-24). For the most part, they are normochromic and normocytic. They may be observed as an artifact, usually as a result of specimen contamination, in which case they will appear in large numbers and will present with evenly dispersed smooth projections and may be referred to as crenated. The terms "crenated cell" and "echinocyte" may be used interchangeably by some reviewers and therefore are not reported. "True" burr cells occur in small numbers and appear irregularly sized with unevenly spaced spicules. They may be seen in uremia, heart disease, cancer of the stomach, bleeding peptic ulcer, immediately following an injection of heparin, and in patients with untreated hypothyroidism. In general, they may occur in situations that cause a change in tonicity of the intravascular fluid (e.g., dehydration and azotemia) or an alteration of the membrane lipid content.² Burr cells may be considered pathological and should be reported.

Acanthocytes (Thorn Cells, Spur Cells)

An **acanthocyte** is defined as a cell of normal or slightly reduced size, possessing 3 to 12 spicules of uneven length distributed along the periphery of the cell membrane. The uneven projections of the acanthocyte are blunt rather than pointed, and the acanthocyte can easily be distinguished from the peripheral smear background because it appears to be saturated with hemoglobin. It appears essentially as a spherocyte with thorns. The MCHC is, however, always in the normal range (Fig. 4-26).

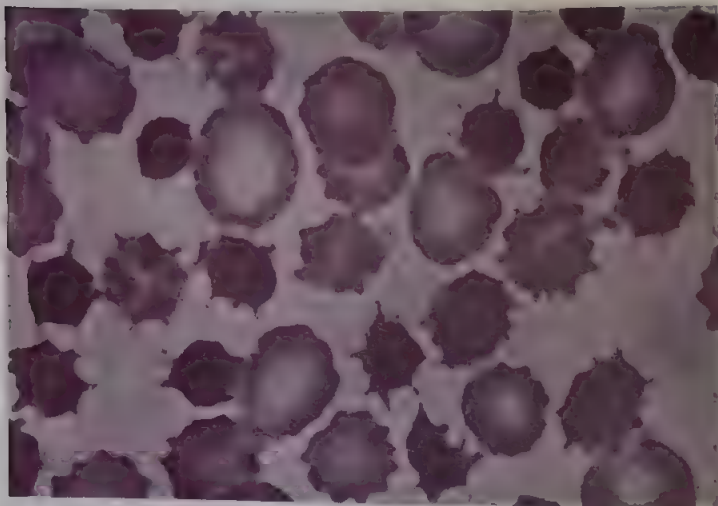


FIGURE 4-26 Note the acanthocytes on this peripheral smear.

ADVANCED CONTENT

Specific mechanisms relating to the formation of acanthocytes are unknown; however, some details about these peculiar cells are of interest. Acanthocytes contain an excess of cholesterol and have an increased cholesterol-to-phospholipid ratio; consequently, their surface area is increased. The lecithin content of acanthocytes is decreased. The only inherited condition in which acanthocytes are seen in high numbers is the rare condition abetalipoproteinemia. Most cases of acanthocytosis are acquired, such as the deficiency of lecithin-cholesterol acyltransferase, which has been well documented in patients with severe hepatic disease. This enzyme is synthesized by the liver and is directly responsible for esterifying free cholesterol; when this enzyme is deficient, cholesterol is increased in the plasma. Acanthocytes may also be seen in myeloproliferative disorders, microangiopathic hemolytic anemia (MAHA), and autoimmune hemolytic anemias. The presence of acanthocytosis in peripheral blood smears remains the hallmark of the clinical diagnosis of most neuroacanthocytosis syndromes, such as chorea-acanthocytosis (ChAc) and McLeod syndrome.¹⁸

The red cell responds to an excess of cholesterol in one of two ways, depending on the balance of other lipids in the membrane. It will become a target cell or an acanthocyte. Once an acanthocyte is formed, it is very liable to splenic

sequestration and fragmentation, and the fluidity of the membrane is directly affected. The most prominent pathologies in which acanthocytes may be observed are listed in Figure 4-27.

Teardrop Cells (Dacrocytes)

Teardrop cells (dacrocytes) appear in the peripheral circulation as tear-shaped or pear-shaped red cells (Fig. 4-28). The extent to which a portion of the red cells form tails is variable and these cells may be normal, reduced, or increased in size. The exact physiological mechanism is unknown, yet teardrop formation from inclusion-containing red cells is well documented. As cells containing large inclusions attempt to pass through the microcirculation, the portion of the cells containing the inclusion cannot pass through and consequently gets pinched, leaving a tailed end. For some reason, the red cell is unable to maintain the discocyte shape once this has occurred.

ADVANCED CONTENT

Teardrop cells are seen most prominently in primary myelofibrosis (see Chapter 19). This type of morphological finding can also be seen in patients with thalassemia syndromes, drug-induced Heinz body formation, iron deficiency, and conditions in which inclusion bodies are formed. They may also be seen in megaloblastic processes as large tear-shaped cells (macroteardrops).

Refer to Figure 4-3 for a composite of abnormal red cell morphology.

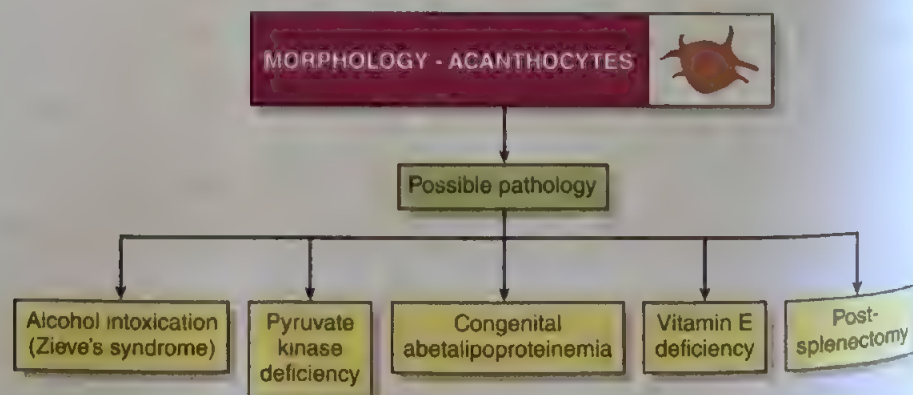
Red Cell Inclusions

Red cell inclusions include Howell-Jolly Bodies, Basophilic Stippling, Pappenheimer Bodies and Siderotic Granules, Heinz Bodies, Cabot Rings, Hemoglobin CC Crystals, Hemoglobin SC Crystals, and Protozoan Inclusions.

Howell-Jolly Bodies

Howell-Jolly bodies (Fig. 4-29) are nuclear remnants containing DNA. They are 1 to 2 μm in size and may appear

FIGURE 4-27 Correlation of acanthocytes to pathological processes.



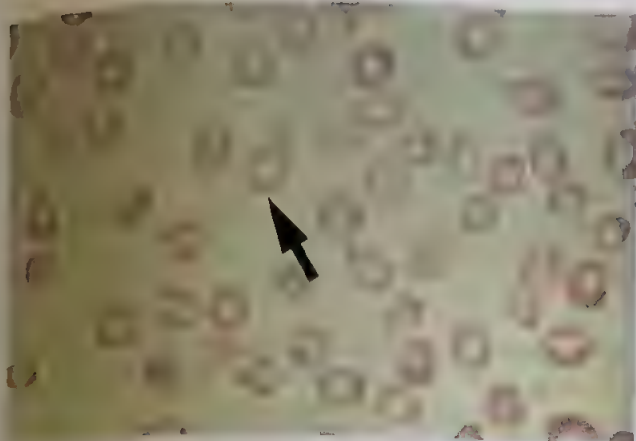


FIGURE 4-28 Teardrop cells (peripheral blood).

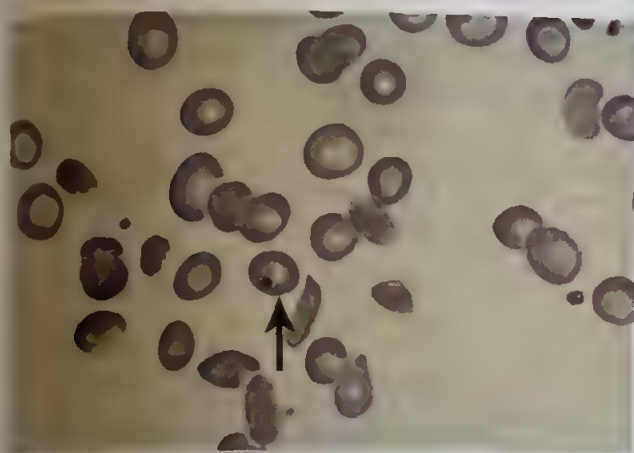


FIGURE 4-29 Howell-Jolly body.

singly or doubly in an eccentric position on the periphery of the cell membrane. They are thought to develop in periods of accelerated or abnormal erythropoiesis. They may be seen in Romanowsky, i.e., Wright's, Giemsa, or supravital stained peripheral smears.

A fragment of the chromosome becomes detached and is left floating in the cytoplasm after the nucleus has been extruded. Under ordinary circumstances, the spleen effectively pits these nondeformable bodies from the cell. However, during periods of erythroid stress, the pitting mechanism cannot keep pace with inclusion formation.

Howell-Jolly bodies may be seen after surgical splenectomy, congenital absence of the spleen, or splenic atrophy after multiple infarctions. They may also be seen in patients with thalassemic syndromes, sickle cell anemia as well as other hemolytic anemias, and in megaloblastic anemias.

Basophilic Stippling

Red cells that contain ribosomes can potentially form stippled cells; however, it is thought that the actual stippling is the result of the drying of cells in preparation for microscopic examination. Coarse, diffuse, or punctate basophilic stippling may occur and consist of ribonucleoprotein and mitochondrial

remnants (Fig. 4-30). These aggregates of ribosomes result from an alteration in the biosynthesis of hemoglobin.

Diffuse basophilic stippling appears as a fine blue dusting, whereas coarse stippling is much more clearly outlined and easily distinguished. Punctate basophilic stippling is a coalescing of smaller forms and is very prominent and easily identifiable.

Stippling may be found in any condition showing defective or accelerated heme synthesis, such as alcoholism, thalassemia syndromes, megaloblastic anemias, and arsenic intoxication. It is also considered a characteristic feature in the diagnosis of lead poisoning. Basophilic stippling may be seen on a Romanowsky or supravital stained peripheral smear. It is important for the reviewer not to confuse stippling with Pappenheimer bodies. The primary differentiation factors are that stippling appears homogeneously over the cell, whereas Pappenheimers tend to appear as clusters in the cell's periphery.

Pappenheimer Bodies and Siderotic Granules

Pappenheimer bodies are also called **siderotic granules** and are small, irregular magenta inclusions seen along the periphery of red cells. They usually appear in clusters, as if they have been gently placed on the red cell membrane. Their presence on a Wright's or a supravital stained peripheral smear is presumptive evidence for the presence of iron. However, the Prussian blue stain is the confirmatory test for determining the presence of these inclusions. These bodies/granules in RBCs are nonheme iron, resulting from an excess of available iron throughout the body. Even though Pappenheimer bodies and siderotic granules are the same inclusion, they are designated differently depending on the stain used. The inclusions are termed "Pappenheimer bodies" when seen in a Wright-stained smear (Fig. 4-31) and "siderotic granules" when seen in Prussian blue or other kinds of iron stain. The explanation for the difference in terminology is that Romanowsky stains (such as the Wright stain) visualize Pappenheimer bodies by staining the protein matrix of the granule, whereas Prussian blue stain is responsible for staining the iron portion of the granule.

Once the presence of siderotic granules has been confirmed by iron stains, the cells in which they are found are

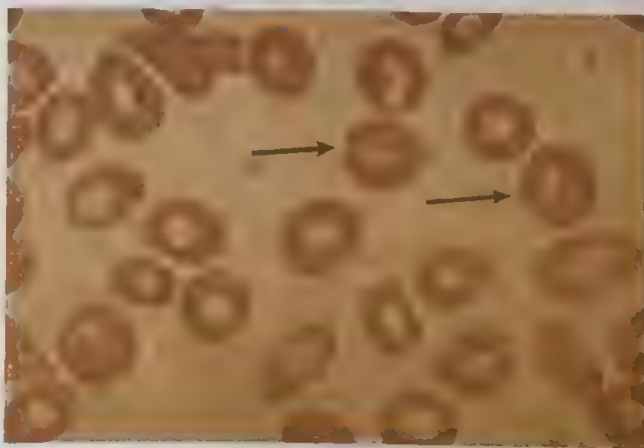


FIGURE 4-30 Note the cells with red cell inclusions: basophilic stippling seen on a peripheral smear in a patient with lead poisoning.

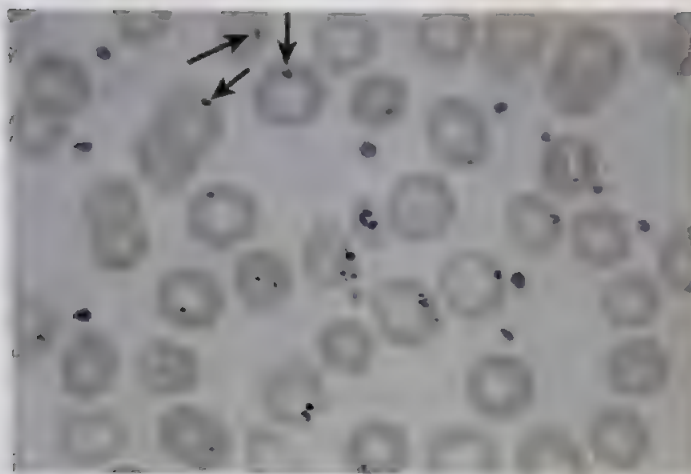


FIGURE 4-31 Pappenheimer bodies (Wright stain).

termed “siderocytes.” Siderocytes containing a nucleus are described as **sideroblasts** and are commonly seen in sideroblastic anemias. Sideroblasts exhibiting numerous siderotic granules found within the mitochondria forming a ring around at least one-third of the nucleus are labeled as pathological ringed sideroblasts. Siderocytes are seen in any condition in which there is iron overloading such as hemochromatosis or hemosiderosis. They may also be seen in the hemoglobinopathies (e.g., sickle cell anemia and thalassemia) and in patients following splenectomy.

Heinz Bodies

Heinz bodies are formed as a result of denatured or precipitated hemoglobin. They are large (0.3 to 2 μm) inclusions that are rigid and severely distort the cell membrane. They can be formed for visualization *in vitro* by incubation with **phenylhydrazine** (a strong oxidizing agent). On initial exposure to phenylhydrazine, small crystalline bodies appear, coalesce, and migrate to an area beneath the cell membrane. This procedure is used before staining with crystal violet or brilliant cresyl blue where the presence of Heinz bodies may be seen on the peripheral smear. Heinz bodies cannot be visualized with Romanowsky stains (Fig. 4-32).



FIGURE 4-32 Heinz body prep; note the appearance of Heinz body inclusions.

ADVANCED CONTENT

Heinz bodies may be seen in the α -thalassemic syndromes, glucose-6-phosphate dehydrogenase (G6PD) deficiency under oxidant stress, and in any of the unstable hemoglobin syndromes (i.e., hemoglobin Köln, hemoglobin Zurich). They may also be seen in red cell injury resulting from chemical insult.

Cabot Rings

The exact physiological mechanism in **Cabot ring** formation has yet to be explained. This structure may represent a part of the mitotic spindle, remnants of microtubules, or a fragment of the nuclear membrane. Cabot rings are found in heavily stippled cells and appear in a figure-eight conformation similar to the beads of a necklace (Fig. 4-33). Cabot rings may be found in megaloblastic anemias, dyserythropoiesis, homozygous thalassemia syndromes, and postsplenectomy. Table 4-5 summarizes abnormal red cell morphologies and associated disease states.

Hemoglobin C Crystals

Hemoglobin (Hb) C crystals may be found in hemoglobin C disease. HbC disease is a mild chronic hemolytic anemia in which the patient is homozygous for the abnormal hemoglobin C.² HbC crystals are formed by the crystallization of the abnormal hemoglobin into one end of the red cell membrane. The crystal forms in a hexagonal shape with blunt ends, leaving the remainder of the cell with the appearance of being empty. These crystals tend to stain dark red and are said to resemble a “bar of gold” and may be referred to as such (Fig. 4-34).

HbC crystals may not always be demonstrated in HbC disease, but their appearance has been found to increase after splenectomy. HbC crystals are not seen in HbC trait (HbAC).

Hemoglobin SC Crystals

Hemoglobin SC (HbSC) crystals may be found on the peripheral smears of patients diagnosed with HbSC disease.

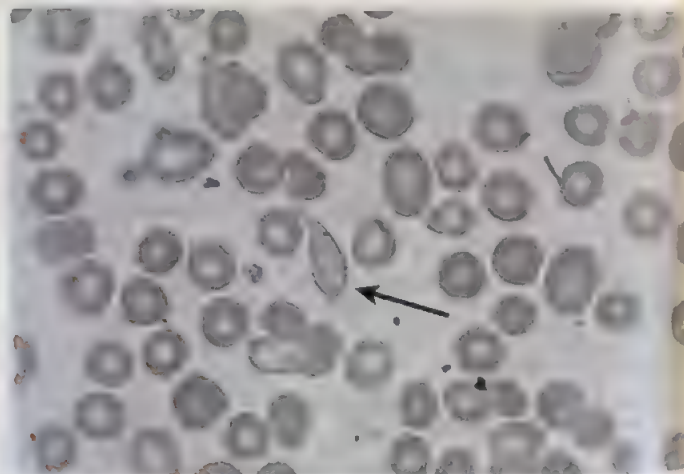


FIGURE 4-33 Note the appearance of a Cabot's ring in the cell at the arrow.

TABLE 4-5 Summary of Abnormal Red Cell Morphologies and Associated Disease States

Abnormal Red Cell Morphologies	Associated Disease States
Microcytes	<ul style="list-style-type: none"> Iron-deficiency anemia Thalassemias Lead poisoning Sideroblastic anemia
Macrocytes	<ul style="list-style-type: none"> Megaloblastic anemias High reticulocyte count Liver disease Myelodysplastic syndromes
Target Cells	<ul style="list-style-type: none"> Liver disease Hemoglobinopathies Thalassemias Sideroblastic anemia
Spherocytes	<ul style="list-style-type: none"> Hemolytic anemias Post-transfusion Hereditary spherocytosis
Elliptocytes	<ul style="list-style-type: none"> Hereditary elliptocytosis Iron-deficiency anemia Thalassemias
Stomatocyte	<ul style="list-style-type: none"> Acute alcoholism Malignancies
Sickle Cells	<ul style="list-style-type: none"> Sickle cell anemia Sickle thalassemia
Acanthocytes	<ul style="list-style-type: none"> Congenital abetalipoproteinemia Vitamin E deficiency Alcohol intoxication Postsplenectomy
Burr Cells	<ul style="list-style-type: none"> Liver disease Renal disease Severe burns Bleeding gastric ulcers
Helmet Cells	<ul style="list-style-type: none"> G6PD deficiency Pulmonary emboli
Schistocytes	<ul style="list-style-type: none"> Disseminated intravascular coagulopathy (DIC) Thrombotic thrombocytopenic purpura (TTP) Hemolytic uremic syndrome Microangiopathic hemolytic anemia
Teardrop Cells	<ul style="list-style-type: none"> Severe anemias Myeloproliferative disorders Pernicious anemia

Hemoglobin SC disease is a chronic hemolytic disorder punctuated by acute painful crisis and diverse chronic organ damage, secondary to the presence of both HbS and HbC.¹⁹ The pathophysiology of the disease is exacerbated by the presence of both hemoglobins, as they tend to exhibit traits that are

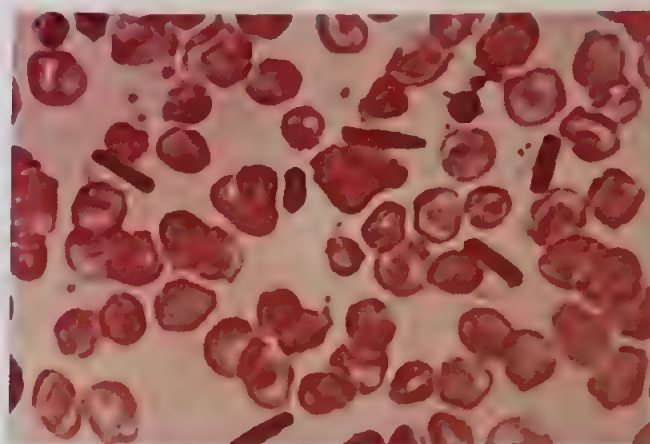


FIGURE 4-34 Note the hexagonal-shaped crystal inclusions in a peripheral smear from a patient with HbC disease. These HbC crystals leave the appearance of the remainder of the cellular cytoplasm as “empty.”

common to each such as sickling from HbS and crystallization from HbC. The result of this combination is the formation of crystals with fingerlike blunt-pointed projections protruding from the cell membrane. The projections have been said to resemble the Washington Monument, and consequently SC crystals may be referred to as “Washington Monument” crystals (Fig. 4-35) (see Chapter 11).

Protozoan Inclusions

Two organisms are briefly discussed in this section because of their tendency to invade the red cells, and the fact that their appearance on a peripheral blood smear is confirmation of infection by the organism. Although only an experienced reviewer would be expected to differentiate these organisms, it is important that all slide reviewers have knowledge of these organisms to recognize their appearance as an abnormality needing further review or testing if incidentally found.

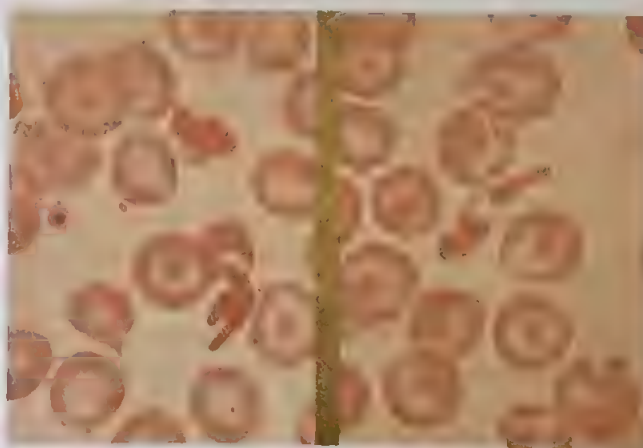


FIGURE 4-35 Note the “fingerlike projections” in this peripheral smear from a patient with HbSC disease. These HbSC crystals are said to resemble the Washington Monument.

ADVANCED CONTENT

All four species of the malaria parasite will invade RBCs. The species include *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium falciparum*, and *Plasmodium ovale* and are transmitted by the *Anopheles* mosquito (see Chapter 14). The parasite may appear in different forms (i.e., ring or troph), and although it is important to the physician for a differential diagnosis and treatment, all reviewers are not expected to be proficient in identification of the specific form. The primary concern is the recognition of the abnormality as a parasite and that it is not confused with normal morphology such as platelets superimposed over red cells.

Babesia microti is also an organism that invades red cells. It is transmitted by tick bites and may appear as ring forms resembling some forms of malaria. The distinguishing feature of *Babesia* is that it also invades blood circulation and on blood smears may appear in groups outside the erythrocyte. Patient symptoms and travel history are also useful in differentiating the two organisms (Fig. 4-36).

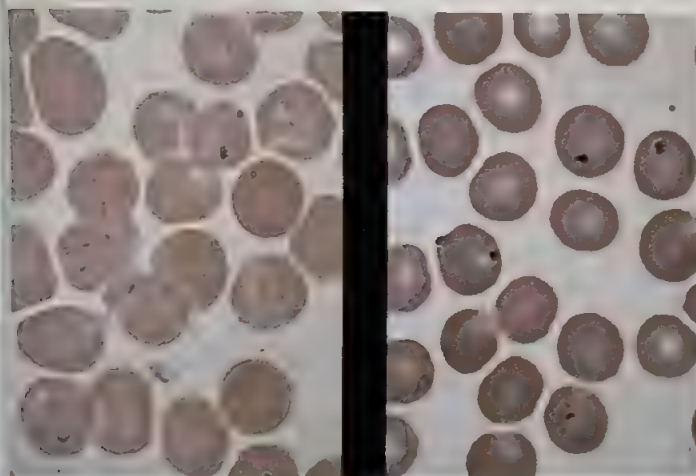


FIGURE 4-36 Comparison of babesiosis (left) and malarial forms (right).

Examination of Platelet Morphology

The normal platelet has several distinctive morphological characteristics. This structure measures approximately 2 to 4 μm , with a discoid shape and even blue granules dispersed throughout a light-blue cytoplasm (Fig. 4-37). In rare instances, one may see megakaryocytic fragments in the peripheral circulation.

ADVANCED CONTENT

A close and thorough examination of platelet morphology provides important information about the patient's hemostatic capability. Variation in platelet morphology may be seen in infiltrative disease of the bone marrow (e.g., primary myelofibrosis or metastatic infiltrates). Large platelets may be seen in

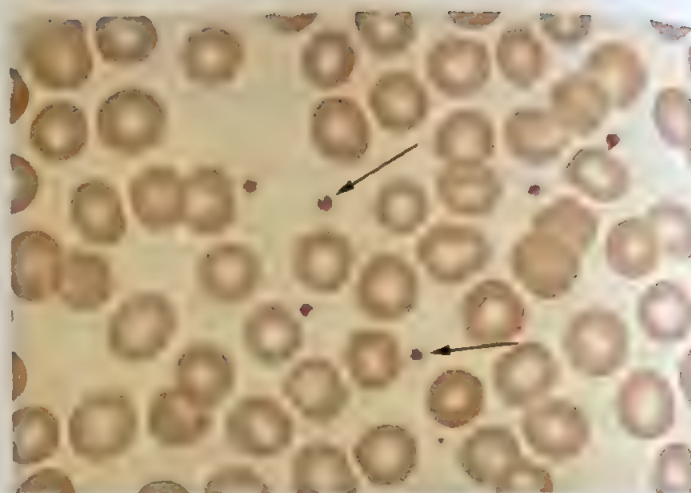


FIGURE 4-37 Normal platelets at arrows.

any disorder associated with increased platelet turnover, such as may occur with idiopathic thrombocytopenic purpura or bleeding disorders. In addition to the elevated platelet count, morphological changes may also occur postsplenectomy.

Slide reviewers should be aware that spurious thrombocytopenia can sometimes occur in samples that are collected with EDTA. Patients that have an antibody to that specific anticoagulant can have platelets that form rosettes and satellite around neutrophils²⁰ and are often flagged by the hematology analyzer as "platelet clumping." Although this is a rare occurrence, it can be resolved by recollecting the peripheral blood sample with sodium citrate as the tube anticoagulant.²¹ A stained slide made from the citrate tube should then be reviewed to confirm the resolution, and the platelet count will need correction before reported. Figure 4-38 shows platelets surrounding a neutrophil. A careful investigation into the patient's clinical presentation along with the delta

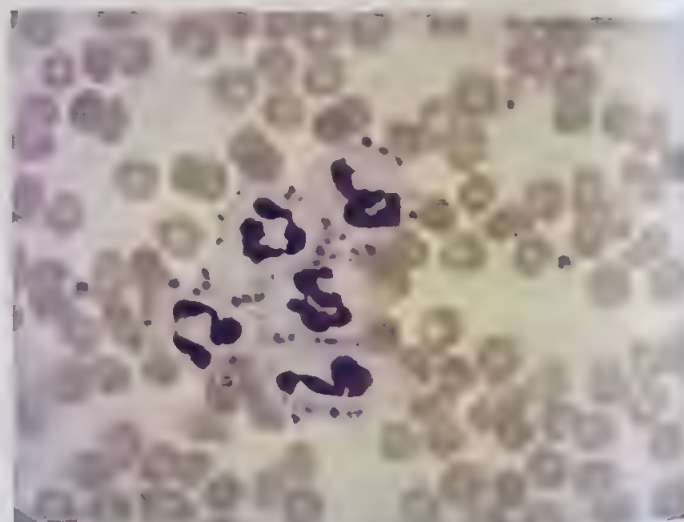


FIGURE 4-38 Note the platelets surrounding the neutrophil (satellitism). Photo courtesy of Birte Hanisch.

check should be considered in these patients before reporting a low platelet count.

CRITICAL THINKING QUESTION

- 4-6 If a clotted sample is submitted for a CBC, would you expect the platelet count to be falsely elevated or decreased? What might be found on peripheral smear?

Examination of White Blood Cell Morphology

The evaluation of white blood cells (WBCs, leukocytes) is performed primarily in response to abnormalities identified by the automated blood cell counter. As with the evaluation of red cell morphology, the technologist must be proficient in the identification of normal WBC morphology to adequately identify morphological abnormalities. Figure 4-39 includes a normal neutrophil as well as a normal lymphocyte. Slide reviewers must be able to recognize the appearance of normal cells, their general size, their shape, and their overall appearance. They must also distinguish between normal granularity and the presence of abnormal inclusions.

Immature White Blood Cells

If immature cells are present, a skilled reviewer should be able to identify the cell line and the stage of maturation. The presence of immature cells is a significant finding, with the greater the immaturity, typically, the more severe the diagnosis.

ADVANCED CONTENT

The presence of more immature forms such as promyelocytes or myeloblasts, in the absences of severe infection, strongly suggests direct marrow architectural involvement. This may indicate an infiltrative, neoplastic, or myeloproliferative process. Regardless of the reason for the appearance of the immature cells, the reviewer should be able to identify the cells accurately. Proper cellular identification at this point may be critical to the patient's diagnosis and subsequent prognosis.

White Blood Cell Morphology

Mature white blood cells may exhibit several morphological changes. In the performance of a slide review and WBC differential, the reviewer should take note of the appearance of the nucleus of the white cells as well as the cytoplasm. Neutrophils tend to exhibit a wider variety of morphological changes than the other cell types. These changes originate in the cytoplasm in response to various pathological processes. The specific alteration may involve the appearance or lack of cytoplasmic inclusions. Severe infections, inflammatory conditions, or other leukemoid reactions may be accompanied

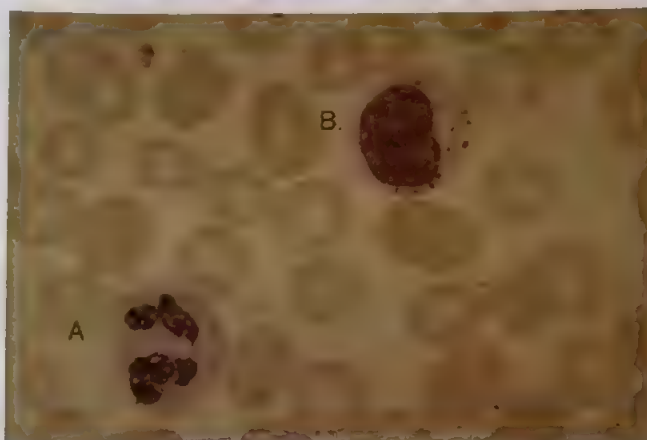


FIGURE 4-39 A, Normal neutrophil, B, normal lymphocyte.

by **toxic granulation**, **toxic vacuolization**, or the presence of **Döhle bodies** (see Chapter 16). Toxic granulation and Döhle bodies are generally considered nonspecific reactive changes, whereas vacuolization strongly indicates a serious bacterial infection. This must also be noted with its importance determined by the physician based on the patient's clinical presentation.

In addition to these morphological changes, severe bacterial infections are also commonly associated with a moderate leukocytosis and a shift to the left in granulocytes. Mild infections are characterized by a slight leukocytosis, with or without the **shift to the left**. Leukocytosis commonly refers to an increase in peripheral blood leukocyte (WBC) concentration of greater than 10,000 cells/ μ L. A shift to the left implies a release of younger granulocytes—specifically bands and metamyelocytes—from the bone marrow storage pool. These particular cell populations may often be observed during an infection or inflammatory process. The degree of leukocytosis or neutrophilia is useful in discriminating among bacterial, viral, or fungal conditions. Because acute infection can rapidly mobilize the neutrophilic nondividing marrow storage pool, the patient usually has a WBC count below 50,000 cells/ μ L (average is 25,000 cells/ μ L). A shift to the left is seen in the peripheral blood smear; however, it is unusual to see cells as immature as myelocytes in the peripheral blood. Fungal infections may also be associated with neutrophilia and an increased WBC count, but a monocytosis is more commonly observed. Viral infections usually are not associated with neutrophilia but rather with lymphocytosis (see Chapter 16).

ADVANCED CONTENT

Leukemoid reactions are characterized by a peripheral neutrophilia that may resemble a chronic leukemia. The WBC count is between 50,000 and 100,000 cells/ μ L, with immaturity observed in one or more cell types. However, a high blast count is not part of the WBC differential picture, which can be helpful in eliminating leukemia as part of

the differential diagnosis. Acute infections, chronic infections such as tuberculosis and chronic osteomyelitis, as well as severe metabolic inflammatory and neoplastic processes have all been associated with leukemoid reactions. Extremely elevated WBC counts (greater than 100,000 cells/ μ L) are more suggestive of a myeloproliferative process (see Chapters 18 and 19), although exceptions have been reported.

The reviewer should also take note of the appearance of the nucleus of the neutrophils and in particular the number of segmentations. The appearance of increased segmentation (normal is three to five lobes) may indicate a megaloblastic process. This is referred to as **hypersegmentation** and is reported when neutrophils contain greater than five lobes or when significant numbers of neutrophils all contain at least five lobes or more. The decreased segmentation should also be noted, as it may be an indication of a benign hereditary condition known as **Pelger–Huet anomaly** or may actually be the result of a leukemic process. This decreased segmentation consists of neutrophils with two lobes or less and is described by the term **hyposegmentation**.

Physiological leukocytosis is defined as an increased WBC count without a shift to the left or any associated morphological changes previously described for granulocytes. This transient condition may be associated with such stimuli as exercise, intense emotional stress, anesthesia, or the administration of epinephrine or glucocorticoids.

WBC Cytoplasmic Inclusions

Cytoplasmic inclusions in WBCs can often indicate a severe clinical presentation, and the competent morphologist must alert the clinician to their presence. Most notable inclusions or cytoplasm variations include toxic granulation and vacuolization, Döhle bodies, blue-green crystals, and morulae from Anaplasmosis.

Toxic Granulation and Vacuolization

Toxic granulation describes medium to large granules that are evenly scattered throughout the cytoplasm of segmented polymorphonuclear neutrophil leukocytes. These granules are seen in metabolically active neutrophilia and are composed of peroxidases and acid hydrolases. Although nonspecific, they may occur in patients with severe bacterial infections, toxemia of pregnancy, vasculitis, or in patients receiving chemotherapy. Toxic vacuolization refers to the round, clear unstained areas that are dispersed randomly throughout the cytoplasm of neutrophils in patients with overwhelming infections.

Döhle Bodies

Additional cytoplasmic inclusions—**Döhle bodies**—are oval, blue, single, or multiple inclusions originating in RNA and are 1 to 3 μ m in diameter. Döhle bodies may be seen in peripheral blood smears of patients with severe infections, in patients with severe burns, in pregnant women, and in patients receiving chemotoxic drugs. In these conditions, they represent toxic changes; however, **Döhle body-like** inclusions are also characteristically observed in certain congenital qualitative WBC

disorders such as the May–Hegglin anomaly and Chédiak–Higashi disorder (see Chapter 24).

Blue-Green Crystals

Refractive blue-green crystals are a rare, yet critical WBC inclusion that have been found in the neutrophils of critically ill patients. These inclusions are most commonly detected in the cytoplasm of neutrophils but can also be found in monocytes. While they are thought to be composed of lipofuscin, their origins are not completely understood. These crystals are acid-fast and have a high lipid content. Formerly called “Green Crystals of Death,” they are associated with elevated lactate levels and multisystem organ failure, specifically the liver.²² The finding and reporting of these crystals are of utmost importance as their presence is an indication of mortality. Figure 4-40 depicts these crystals.

Anaplasmosis

Not to be confused with the aforementioned blue-green crystals, an infection with the bacterium *Anaplasma phagocytophilum* can cause similar-appearing intracellular parasitic inclusions in WBC. Figure 4-41 shows this cytoplasmic inclusion called **morulae** (clumps of blue-grey stained bacteria).²

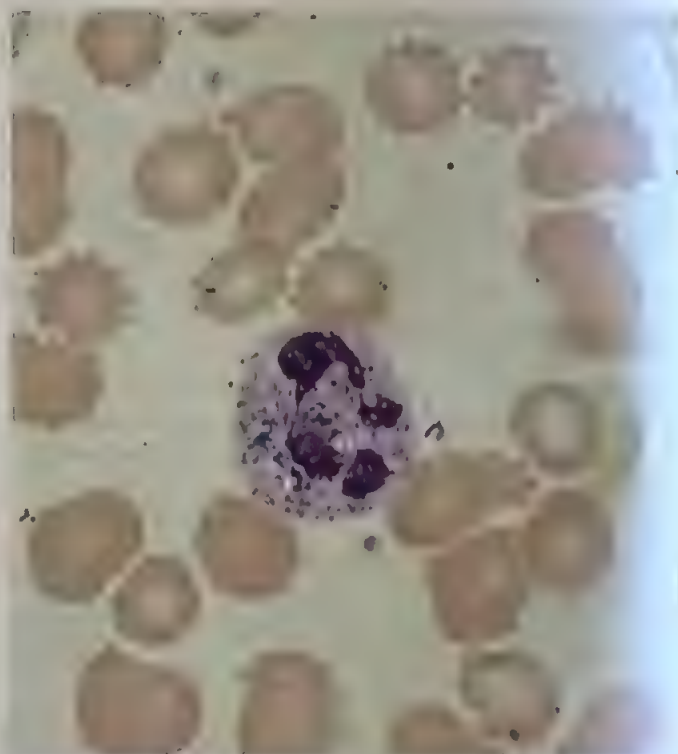


FIGURE 4-40 Blue-green crystals in a neutrophil. Photo courtesy of Angela Bottoms-Popken.



FIGURE 4-41 Morulae seen in a neutrophil.

ADVANCED CONTENT

Human Granulocytic Anaplasmosis was formerly called Ehrlichiosis and is a disease most often transmitted by the *Ixodes* tick. Patients present with fever, decreased WBCs, a

left-shift, decreased platelets, and elevated liver enzymes.²⁴ Confirmation of the disease can be made with PCR or antibody testing.

SUMMARY CHART

- On an automated cell counter, a flag is a signal that a significant abnormality may be present in the sample.
- A delta check is the comparison of current results with previous results.
- Reflex testing is when current results indicate the need for additional testing and those analysis are automatically ordered for evaluation.
- Peripheral smear analysis should always start with review of red cell distribution at low-power 10 \times magnification.
- WBC differential and RBC evaluation should take place under oil immersion 100 \times .
- Normal RBC morphology includes a discoid shape 7–9 μ m in diameter.
- Microcytes are associated with an MCV of less than 80 fL and are seen in IDA, thalassemias, sideroblastic anemias, and anemia of chronic inflammation.
- Macrocytes are associated with an MCV of more than 100 fL and are seen in megaloblastic and nonmegaloblastic processes.
- A variation in cell size is termed anisocytosis.
- A variation in cell shape is termed poikilocytosis.
- Normal red blood cell distribution is when red cells are close but not touching or overlapping one another.
- Macrocytes are associated with high reticulocyte counts and liver disease; oval macrocytes are associated with megaloblastic processes.
- Red cells that appear polychromatophilic on a Wright's-stained smear would appear as reticulocytes on a supravital stain.
- Spherocytes are associated with hereditary spherocytosis and an MCHC that is greater than 36% in many cases.
- Spherocytes may also be seen in autoimmune hemolytic anemia (AIHA) and post-transfusion.
- There are two types of sickle cells, irreversible sickle cells (ISCs) and oat-shaped, reversible sickle cells that are both commonly seen in HbS disease.
- Any regenerative red cell process can result in inclusions such as Howell–Jolly bodies, basophilic stippling, and Pappenheimer bodies.
- Howell–Jolly bodies, Pappenheimer bodies, and basophilic stippling may be seen in peripheral smears stained with both Romanowsky type stain (i.e., Wright's, May–Grünwald) and supravital stain (i.e., new methylene blue, brilliant cresyl blue).
- Siderotic granules and Pappenheimer bodies are basically the same inclusion. The differentiating factor is that on an iron stain the inclusions are known as siderotic granules whereas on Wright's stain they are known as Pappenheimer bodies.
- Howell–Jolly bodies are seen in patients postsplenectomy.
- Helmet cells are seen in patients with G6PD syndrome and occur as a result of Heinz body formation.
- Heinz bodies cannot be seen on a Wright-stained peripheral smear.
- Abnormal platelet morphology includes lack of granulation, giant platelets, and megakaryocytic fragments.
- Platelet satellitism may cause pseudo thrombocytopenia in samples collected in EDTA.
- Abnormal white cell morphology includes the presence or absence of cytoplasmic granulation, presence of cytoplasmic inclusions, presence of cytoplasmic vacuolization, as well the appearance of the cellular nucleus.
- Hyposegmentation describes decreased neutrophil segmentation (≤ 2 lobes).
- Hypersegmentation describes increased neutrophil segmentation (≥ 5 lobes).

CASE STUDY 4-1

A 1-year-old African American child is brought to the emergency department by her mother because the child had no appetite and had not eaten in the last 2 days. Additional information from the mother described a normally happy child who had recently become restless and irritable. She was learning to walk but now would not even attempt standing. She has had a low-grade temperature which is now elevated to 102°F. On examination, there is a definite yellow tinge to the sclerae, and the spleen is palpable. Also noted was a spindle-shaped deformity of two fingers on the right hand that were swollen and obviously painful to the touch. The CBC results were as follows:

CBC Results	Patient Results	Reference Range
WBC	$17.0 \times 10^9/L$	$6.0-17.5 \times 10^9/L$
RBC	$2.4 \times 10^{12}/L$	$3.8-5.5 \times 10^{12}/L$
Hgb	7.5 g/dL	12-14.5 g/dL
Hct	22.0%	30%-43%
MCV	92 fL	80-100 fL
RDW	18%	11.5%-14.5%

WBC Differential		RBC Morphology	
40%	Granulocytes	Anisocytosis	3+
58%	Lymphocytes	Poikilocytosis	3+
2%	Monocytes	Hypochromia	1+
		Macrocytosis	1+
		Microcytosis	1+
		Schistocytes	2+
		Sickled cells	1+
		Targets	1+
		Polychromasia	1+

Additional Tests Ordered

Reticulocyte count	13%
Sickledex	Positive
Hgb electrophoresis	Hgb SS pattern

DISCUSSION

Sickle cell disease is a hereditary disease that results in a chronic moderate to severe hemolytic anemia. The disease is characterized by the substitution of valine from the normal glutamic acid at the sixth position in the β -chain, resulting in an abnormal hemoglobin that polymerizes when exposed to low oxygen-tension conditions. This polymerization results in the formation of sickle-shaped cells that are capable of temporarily or permanently blocking microcirculation, and the resulting stasis may lead to hypoxia and ischemic infarcts of various organs.

The disease is not evident at birth and does not manifest itself until the gamma chains of the newborn are replaced by β -chains after 3 to 6 months of life.¹⁶ Clinical manifestations may be divided into acute and chronic episodes. Acute problems result from a vaso-occlusive crisis termed "sickle cell crisis." This "crisis" typically includes an acute hemolytic episode as well. Patients in sickle cell crisis present

with acute pain, fatigue, and possibly jaundice. All three of these symptoms were present in our case study, as was evidence of some form of anemic process.

Chronic manifestations of sickle cell disease usually appear after mid-childhood.¹⁶ These include disturbances in growth and development, bone and joint disease, and organ damage involving mostly all of the organ systems of the body at some point during the process of the disease.

QUESTIONS

1. Are the morphological findings on the blood film compatible to the results from the analyzer?
2. Did the other tests ordered confirm a probable diagnosis?
3. Is the reticulocytes count useful?

ANSWERS

1. Note: Manual differential and morphology review performed due to flagging of analyzer results (Fig. 4-42). Morphology results correlate with the values from the instrument, for example, the RDW is elevated, and this is reflected on the smear as 3+ anisocytosis.
2. Yes. The sickledex is a screening test performed on whole blood and is positive in both sickle cell disease and sickle cell trait. The confirmatory test is the hemoglobin electrophoresis, which will differentiate abnormal hemoglobin traits from abnormal hemoglobin disease. In this case, the presence of the SS pattern is diagnostic of Hgb S disease, which will result in sickle cell anemia.
3. Yes. The reticulocytes count is a valid indicator of bone marrow performance. In cases of sickle cell crisis, in which the nonfunctional sickle cells are being destroyed, a properly functioning marrow should accelerate the hematopoietic process. The 13% reticulocytes count is indicative of a proper response by the bone marrow to the hemolytic process the patient was experiencing.

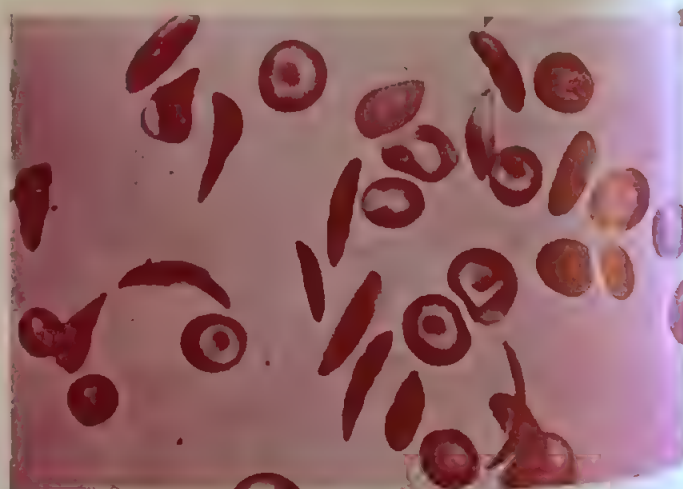


FIGURE 4-42 Case study; note abnormal red cell morphology.

CASE STUDY 4-2

A CBC is performed on a sample in the hematology laboratory. The CBC results were as follows:

CBC Results	Patient Results	Reference Range
WBC	$9.0 \times 10^9/L$	$6.0-17.5 \times 10^9/L$
RBC	$4.2 \times 10^{12}/L$	$3.8-5.5 \times 10^{12}/L$
Hgb	12.2 g/dL	12-14.5 g/dL
Hct	36.0%	30%-43%
MCV	102 fL	80-100 fL
RDW	14.7%	11.5%-14.5%

WBC Differential

90%	Granulocytes
9%	Lymphocytes
1%	Monocytes

QUESTIONS

- Based on the results from the analyzer, what should you expect to see on the peripheral smear regarding RBC morphology?

- With such a high percentage of neutrophils, what should these cells be evaluated for closely on peripheral smear review?

ANSWERS:

- With a high MCV, there should be macrocytes present in the peripheral blood smear. Additionally, because the RDW is normal, it is likely that most of the RBCs present are macrocytes (meaning there is not a large amount of RBC variation).
- A high percentage of neutrophils could indicate an inflammatory or infectious condition. It is important to look for additional signs such as toxic granulation or Döhle bodies to aid in the differential diagnosis of these possible conditions.

REVIEW QUESTIONS

- Which of the following results will prompt a manual slide review?
 - Delta check
 - High RBC count
 - High WBC count
 - Normal platelet results
- How many oil immersion fields (OIFs) should be evaluated to determine cell morphology?
 - 8-10 OIFs
 - 2-4 OIFs
 - 3-5 OIFs
 - 1-6 OIFs
- A normocyte has an average diameter of:
 - 2-4 μm
 - 5-6 μm
 - 7-8 μm
 - 9-10 μm
- A prominent morphological clue when suspecting lead poisoning is the presence of which of the following on a peripheral smear?
 - Heinz bodies
 - Target cells
 - Siderotic granules
 - Basophilic stippling
- In which of the following disease states would you expect to find oval macrocytes on the peripheral smear?
 - Iron deficiency anemia
 - Lead poisoning
 - Megaloblastic anemia
 - Hereditary spherocytosis
- In which of the following would you expect to find hyposegmented neutrophils?
 - Liver disease
 - Megaloblastic anemia
 - Sickle cell anemia
 - Pelger-Huet anomaly
- An abnormal erythrocyte seen in liver disease, hemoglobinopathies, and thalassemias and is characterized by the "bull's eye" area is known as a:
 - Stomatocyte
 - Target cell
 - Schistocyte
 - Hypochromic cell
- Morphological abnormalities found in cases of severe burns, microangiopathic hemolytic anemias, and disseminated intravascular coagulation (DIC) are:
 - Schistocytes
 - Crenated cells
 - Ovalocytes
 - Stomatocytes

REVIEW QUESTIONS—cont'd

9. Oat-shaped cells may be associated with:
 - a. Myelofibrosis
 - b. Hereditary spherocytosis
 - c. Burns
 - d. Sickle cell anemia
 10. How would a cell be classified that has a diameter of 9 μm and an MCV of 104 fL?
 - a. Macrocytic
 - b. Microcytic
 - c. Normal
 - d. Either normal or slightly microcytic
 11. Abnormal platelet morphology may be observed most prominently in:
 - a. Primary myelofibrosis
 - b. Anemia of chronic disorders
 - c. Hereditary spherocytosis
 - d. Septic shock
 12. Which type of red cell inclusion is a DNA remnant?
 - a. Heinz body
 - b. Howell–Jolly body
 - c. Pappenheimer body
 - d. Cabot ring
 13. Which of the following is considered a microcytic/hypochromic anemia?
 - a. Autoimmune hemolytic anemia
 - b. Pernicious anemia
 - c. Iron deficiency anemia
 - d. Megaloblastic anemia
 14. A hypersegmented neutrophil may be seen in which of the following anemias?
 - a. Iron deficiency
 - b. Megaloblastic
 - c. Autoimmune hemolytic anemia
 - d. Anemia of chronic disorders
 15. Precipitates of denatured hemoglobin found primarily in patients with hemolytic anemia resulting from oxidant stress describe:
 - a. Howell–Jolly bodies
 - b. Heinz bodies
 - c. Basophilic stippling
 - d. Pappenheimer bodies
 16. Pappenheimer inclusions are formed from:
 - a. Excess α -chains
 - b. Excess β -chains
 - c. Excess iron
 - d. Oxidant stress
 17. Which of the following RBC inclusions results from an acceleration in hemoglobin biosynthesis and consists of RNA?
 - a. Howell–Jolly bodies
 - b. Heinz bodies
 - c. Basophilic stippling
 - d. Pappenheimer bodies
 18. A release of younger WBC from the bone marrow in response to an infection or inflammatory process is termed:
 - a. Shift to the left
 - b. Leukocytosis
 - c. Toxic vacuolization
 - d. Right shift
 19. Hypersegmentation is reported when the number of lobes of neutrophils exceed:
 - a. 1
 - b. 2
 - c. 3
 - d. 5
 20. Blue oval cytoplasmic inclusions found in the neutrophils of patients with severe burns and receiving chemotoxic drugs are:
 - a. Cabot rings
 - b. Döhle bodies
 - c. Anaplasmosis
 - d. Howell–Jolly bodies
 21. Which of the following should be evaluated for clumping on a stained peripheral smear?
 - a. NRBCs
 - b. RBCs
 - c. Platelets
 - d. Neutrophils
- The following answer pool is used for items 22 to 36 (Match)
- | | | |
|-------------------|---------------------|-----------------|
| a. Anisocytosis | f. Polychromasia | k. Schistocytes |
| b. Poikilocytosis | g. Microspherocytes | l. Rouleaux |
| c. Hypochromasia | h. Target cells | m. Acanthocytes |
| d. Microcytic | i. Stomatocytes | n. Dacrocytes |
| e. Drepanocyte | j. Blister cells | o. Echinocytes |
- _____ 22. RBCs with a large area of central pallor
 - _____ 23. Variation in the size of the red blood cells
 - _____ 24. Also known as codocytes
 - _____ 25. RBCs appearing stacked on each other

REVIEW QUESTIONS—cont'd

26. MCV of 65%
27. RBCs with a mouthlike central pallor
28. RBCs without an area of central pallor
29. RBCs with evenly distributed spicules on the membrane
30. RBC fragments
31. RBCs appearing bluish in color
32. Variation in the shape of the RBCs
33. Congenital abetalipoproteinemia
34. The formation of a vacuole in an RBC "trapped" by fibrin
35. Formed when an RBC with an inclusion squeezes out of a tight space
36. Seen in HbS disease

See answers at the back of this book.

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Quality Management in the Hematology Laboratory

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CHAPTER OUTLINE

Quality Management

Legal Implications
Quality Management Plans
Quality Approaches
Quality System Essentials

Quality Assurance and Quality Control

Key Definitions
General Quality Assurance Control
Activity Guidelines

Preanalytical, Analytical, and
Postanalytical Factors in Testing
Accuracy, Precision, and Error
Method Validation
CLIA Minimum Quality Control
Requirements
Levy–Jennings Graphs
Westgard MultiRule Quality Control
Peer Group Quality Control

Hematology Laboratory Applications

Quality Plan Example
Method Validation Studies
Quality Control
Case Study 5–1
Case Study 5–2
Review Questions
References

LEARNING OBJECTIVES

At the end of this chapter the learner should be able to:

- | | |
|---|--|
| <p>5-1 Discuss how quality policies and processes are integrated into the laboratory to create a quality management system.</p> <p>5-2 Describe the role of the Centers for Medicare & Medicaid Services (CMS) in regulating laboratory testing.</p> <p>5-3 List and describe the seven principles of a quality management system.</p> <p>5-4 Discuss when and how the following quality approaches are used:</p> <ol style="list-style-type: none"> a. Benchmarking b. Lean c. Six Sigma d. Root Cause Analysis (RCA) e. Failure Mode & Effects Analysis (FMEA) f. Define, Measure, Analyze, Improve, and Control (DMAIC) <p>5-5 Explain the significance of each of the Quality System Essentials (QSE).</p> <p>5-6 Discuss the specimen Path of Workflow in the preexamination (preanalytical), the examination (analytical), and the postexamination (postanalytical)</p> | <p>stages and how quality processes are critical to quality patient results.</p> <p>5-7 Define accuracy, precision, and error as they apply to test results.</p> <p>5-8 Explain standard deviation and how it is related to the distribution of results about the mean.</p> <p>5-9 Outline the process for method validation, including the various experiments required to determine the reportable range and the reference range.</p> <p>5-10 Describe the purposes of QC matrix and assayed vs unassayed QC as quality control materials.</p> <p>5-11 Discuss the importance of Delta Checks for test result integrity and patient safety.</p> <p>5-12 Describe a Gaussian distribution and how it applies to quality control.</p> <p>5-13 Diagram examples of a trend and shift from a Levy–Jennings graph.</p> <p>5-14 Discuss the Westgard MultiRules decision criteria and how they are used to determine whether a quality control run is acceptable.</p> <p>5-15 Describe when a method validation study is necessary.</p> |
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Clinical laboratories provide essential information for effective, quality patient care. Health-care practitioners use laboratory test results for screening, diagnosis, treatment, and ongoing monitoring of disease. At one time, the level of laboratory quality was mainly associated with analytic errors; that is, if the laboratory was generating perceived good results, that was considered enough. Since the introduction of the Clinical Laboratory Improvement Act (CLIA)¹ in 1988, the laboratory quality system has continuously evolved to become a more comprehensive, overarching quality management program that includes not only analytic errors but all errors in laboratory medicine.

Quality policies and processes are integrated in all areas of the laboratory, beginning with the preexamination phase, through the examination phase, to the postexamination phase. As the quality management system has matured, patient outcomes, now considered part of the postexamination phase, are included in the evaluation of what defines quality health-care delivery.

Quality Management

Quality Management (QM) includes all processes that affect the quality of a laboratory test result from beginning to end. A quality test result is accurate and precise, gives the clinician the information to make a medical decision, and is within a reasonable cost (Fig. 5-1). How does a laboratorian ensure quality results? Processes are designed with quality built into every step of the way to comply with regulatory guidelines and best practice models. Quality is everyone's responsibility. With the rapid development of technology, the expanding test menu, and the vast number of tests available to order, ensuring the correct test is selected to provide maximum benefit to the patient is critical. The manner of collection of the specimen, analysis of the specimen, acceptance of results, and verification the clinician receives accurate results with minimal delay are just a few of the variables to consider for ongoing continuous improvement.

Legal Implications

Laboratories and their personnel have potential to incur legal costs when quality management procedures are not instituted or followed. When a laboratory professional fails to adhere

to the policies and standard operating procedures, errors may occur that lead to adverse consequences for patients. In general, laboratories are more likely to be liable for regulatory violations than to be sued for negligence or other legal reasons. However, litigation can occur, and individual laboratory technical personnel may be held liable for harm if the patient can show that the individual acted negligently.² Errors in the laboratory have direct consequences to patient care as well as ultimately patient outcomes. Medical errors also directly affect public confidence and harm the reputation of a hospital or medical center.

Quality Management Plans

The Centers for Medicare & Medicaid Services (CMS) regulate all laboratory testing (except research) performed on humans in the United States through the **Clinical Laboratory Improvement Amendments of 1988 (CLIA)**. According to CLIA's *Survey Procedures and Interpretive Guidelines for Laboratories and Laboratory Services (Appendix C) Part 493, Laboratory Requirements*,³ all laboratories that perform patient testing must have a quality management plan.

To assist laboratories, CMS has identified accreditation organizations that meet or exceed the CLIA requirements and have deemed status for inspecting laboratories. The College of American Pathologists (CAP), The Joint Commission (TJC), and AABB are three examples. These accrediting bodies have resources available to assist laboratories in creating a quality program. The CAP, TJC, and AABB have incorporated regulations from CLIA into their accreditation checklist questions and standards. Another resource for quality management is the Clinical and Laboratory Standards Institute (CLSI). CLSI is a not-for-profit organization committed to advancing excellence in laboratory medicine. The CLSI Standards and Guidelines are developed by an international group of subject matter experts with a vast knowledge of regulatory requirements. These individuals work together to create consensus documents that may be used by laboratories, both in the United States and worldwide.

Laboratory leadership can decide how they would like to implement their Quality Management System. The primary objective should be patient safety based on quality test results. The seven QMS principles⁴ are:

1. **Customer focus:** Meeting customer requirements and striving to exceed customer expectations.
2. **Leadership:** Leaders at all levels must work together to create an environment in which people are engaged in attaining laboratory quality goals.
3. **Engagement of personnel:** There must be competent and engaged personnel throughout the organization to create and sustain the quality initiatives.
4. **Process approach:** The QMS interrelated processes work as a system enabling an effective and efficient laboratory.
5. **Improvement:** Creating a system or process for ongoing awareness for continuous improvement in laboratory operations.
6. **Evidence-based decision-making:** Analysis and evaluation of patient and test data, identifying laboratory best practices, and possibly incorporating Diagnostic

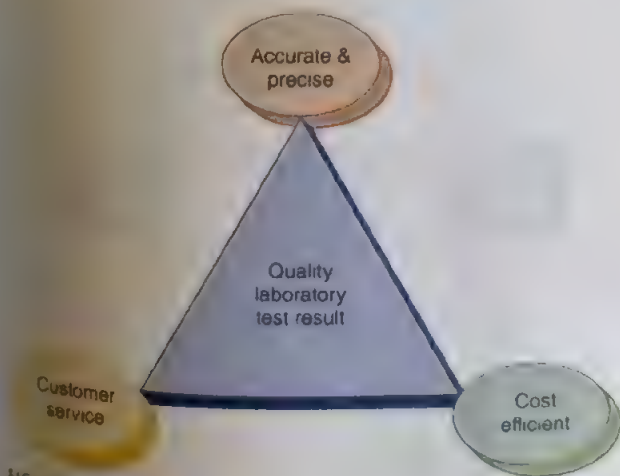


FIGURE 5-1 The elements of a quality laboratory result.

Management Teams (DMT) for clinician consultation for improved patient outcomes.

• **Relationship management:** Creating and sustaining relationships with medical providers and practitioners.

Quality Approaches

To get started, a variety of quality tools are available to assist with gauging how your laboratory is currently functioning and identifying areas for improvement.

Benchmarking

Laboratory benchmarking is the practice of comparing the individual laboratory processes and performance metrics to industry best practices from other laboratories. Published surveys are available with multihospital studies to evaluate how the laboratory is performing compared with its peers.³

Root Cause Analysis

Root Cause Analysis (RCA)⁶ requires insight and understanding of how a process works. RCA examines what happened, how it happened, and why it happened. Various models may be used to perform RCA. One of the more common tools is a method referred to as the Five Whys. The problem is investigated by asking why repeatedly until all the possible reasons for the problem are revealed.

Lean

Lean is a process improvement tool to identify nonvalue-added activities considered as waste.⁷ In health-care settings, Lean has helped reduce turnaround times and streamline and standardize processes throughout the laboratory. The seven types of waste, with examples, are:

1. **Defects:** Errors in laboratory results or reports, missing reports, late reports.
2. **Waiting:** Laboratory personnel are waiting for the patient or the next batch of samples at receiving or testing.
3. **Extra processing:** Rework, such as redraws and retesting that occur because of defects in the original effort.
4. **Transport:** Unnecessary movement of samples, supplies, paper, or staff, and retrieval of lost, moved, or misplaced items needed in the work.
5. **Motion:** Extra steps taken by staff to accommodate workarounds arising from inefficient layout of instrumentation, supplies, storage, or information.
6. **Inventory:** Extra inventory of laboratory reagents, supplies, paper, and other materials that are assets and not directly required for the current work.
7. **Overproduction:** Performing unnecessary laboratory testing for any reason.

Six Sigma

The **Six Sigma** approach focuses on reducing waste by reducing variation.⁸ The term "Six Sigma" refers to a calculation for the Sigma scale; a quality process would have a Sigma score between 3 and 6 Sigma. To achieve Six Sigma (excellent quality), a process must not produce more than 3.4 defects per million opportunities. Many organizations, from the government to private and public companies, are now using Six Sigma practices.

Failure Mode and Effects Analysis

Failure Mode and Effects Analysis (FMEA) is a risk management tool used in health care to identify possible failures of a product or service and then determine the occurrence rate, severity, and detectability of a failure. For example, a test result would be the product, and a failure could be an incorrect test result that could potentially harm the patient. Multiplying the rate of severity of harm by the frequency that the harm might occur by the detectability of the error provides the risk priority number (RPN). (Risk Priority Number = Severity × Occurrence × Detection.) The higher the RPN, the greater the risk to the patient.

Define, Measure, Analyze, Improve, and Control

Define, Measure, Analyze, Improve, and Control (DMAIC) is a five-phase method for improving existing process problems with unknown causes. The define phase is identifying the problem; the measure phase involves collecting the data around the problem; the analyze phase begins with understanding the problem; the improve phase involves designing an intervention; and the control phase ensures the intervention is working and sustained (Fig. 5-2).

Quality System Essentials

The **Quality System Essentials (QSEs)**⁹ are a road map for how a laboratory establishes a Quality Management System (QMS). The QSEs were developed by CLSI and use both national and international regulatory and accreditation requirements. Following the recommended sequence of the QSEs provides an organized way to create an effective quality management system.

The sequence of the QSEs:

1. Organization and Leadership
2. Customer Focus
3. Facilities and Safety Management
4. Personnel Management
5. Supplier and Inventory Management
6. Equipment Management
7. Process Management
8. Documents and Records Management

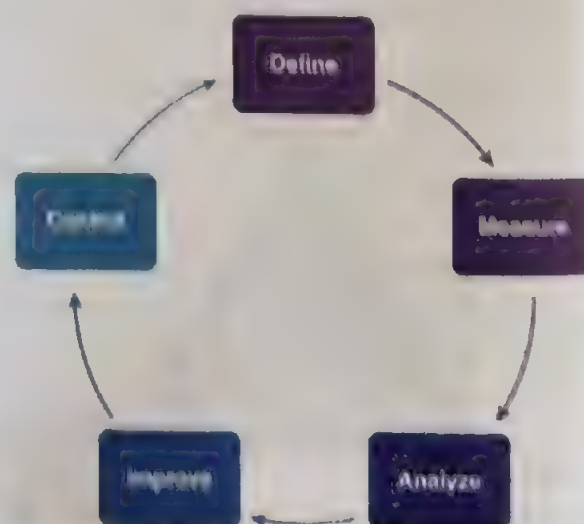


FIGURE 5-2 DMAIC diagram.

9. Information Management
10. Nonconforming Event Management
11. Assessments
12. Continual Improvement

W. Edward Deming, who helped establish modern day quality principles, said, "Doing your best is not good enough. You have to know what to do. Then do your best." The QSEs outlines what the laboratory needs to do for best performance (Fig. 5-3).

Organization and Leadership

To create and sustain a quality system, the laboratory leadership must be committed to quality and take responsibility for the design and implementation of the QMS. This involves developing quality policies, processes, and procedures, and ensuring there are resources for budgets and staff hiring and training. Starting with the creation of an organizational chart and delegating responsibilities is critical so laboratory personnel and regulatory assessors and inspectors know who is in charge.

Customer Focus

The customer is the recipient of a product or service. Laboratory professions should consider who is receiving the service. Every laboratory test and the format of that result should have meaning to the health-care provider, who will then translate the result into an action for a patient. Proper phone etiquette, cooperation among laboratory departments, and calling critical results promptly are examples of good customer service. Periodically surveying the customers is not only fulfilling regulatory requirements but also ensuring the needs of customers are being met.

Facilities and Safety Management

Each laboratory should establish and maintain a facility that has adequate space (for both testing and storage), efficient workflow design (including emergency power proved for essential laboratory equipment), and environmental controls

for temperature, humidity, and ventilation conditions. To support the safety of all personnel, there must be accommodations and access for individuals with disabilities, protection of employees by providing adequate personal protective equipment (PPE), protection of patients and visitors from hazards, and emergency response systems. Safety management includes documented training for PPE, chemical hygiene, fire extinguisher use, and safety audits.

Personnel Management

Employees are the most valuable asset of any organization. Laboratory management must define the job qualifications and job descriptions for each laboratory role, determine educational requirements, provide training, and ensure competency. Assessment of competency for testing personnel must occur after initial training of the employee, at least semiannually during the first year the employee is testing patient specimens, and then annually.

The following six procedures are the minimal regulatory requirements for assessment of competency for all personnel performing laboratory testing:¹⁰

1. Direct observations of routine patient test performance, including patient preparation, if applicable, specimen handling, processing, and testing
2. Monitoring the recording and reporting of test results
3. Review of intermediate test results or worksheets, quality control records, proficiency testing results, and preventive maintenance records
4. Direct observations of performance of instrument maintenance and function checks
5. Assessment of test performance through testing previously analyzed specimens, internal blind testing samples, or external proficiency testing samples
6. Assessment of problem-solving skills

In addition to competency, conducting annual performance evaluations provides useful feedback to the employee, promotes good communication, and contributes to the employee's professional development. Encouraging and supporting continuing education validates the individuals' worth to the laboratory and promotes the importance of the medical laboratory profession.

Supplier and Inventory Management

Purchasing equipment, materials, and other products requires processes to ensure the suppliers can meet laboratory expectations. Qualifying and selecting suppliers involves identifying laboratory needs, formalizing agreements or contracts, and developing a process for purchasing. Inventory management includes identification and tracking of critical materials and services. Monitoring and rotating inventory for usage and expiration dates minimizes waste. Purchasing sequestered lot numbers of reagents is desirable to reduce parallel testing time and money, and to ensure a more stable testing environment.

Equipment Management

When considering the purchase of new equipment, the following should be considered: cost, accuracy, precision, risk, usefulness, ease-of-operation, maintenance schedule, consumables

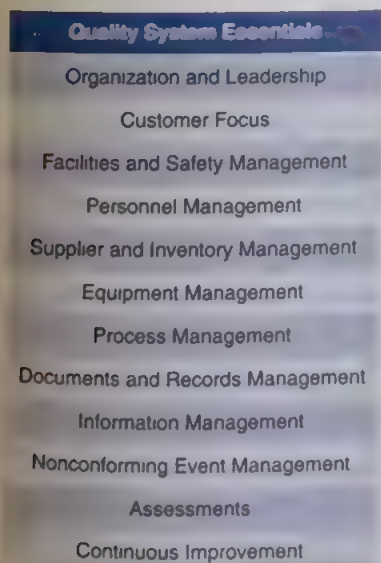


FIGURE 5-3 The 12 Quality Systems Essentials (QSEs).

required, and test menu. The laboratory must have an equipment qualification process that includes **installation qualification (IQ)**, **operational qualification (OQ)**, and **performance qualification (PQ)**. The manufacturer often performs the IQ and verifies the new equipment is functioning as expected. Laboratory personnel and the manufacturer perform the OQ to ensure the equipment is operational and includes powering up the equipment and various function checks.

The final qualification is performance and is the responsibility of laboratory personnel. The purpose of the PQ is to confirm the equipment is producing results under a wide range of normal operating conditions. The laboratory sets the criteria for result acceptability. The laboratory personnel perform the PQ, and the test results must fall within the acceptability criteria detailed in the validation plan.

Equipment management necessitates that the laboratory has a process to maintain files and records that include the IQ, OQ, PQ, maintenance, calibration, problems, service, and repairs, and finally decommission and final disposition.

Process Management

Process management is where most laboratories concentrate their quality practices. The laboratory needs to identify the **path of workflow** (preexamination, examination, and postexamination) to monitor for every laboratory test, procedure, or service. Building quality requirements into work processes will ensure the production of quality results. Procedure manuals, process maps, and flow charts document and communicate process requirements and provide consistent instruction on test performance and recording results. Figure 5-4 is an example of a flowchart for performing a sedimentation rate procedure. Process validation and verification is necessary for all laboratory processes and applies to testing, reagents, equipment,

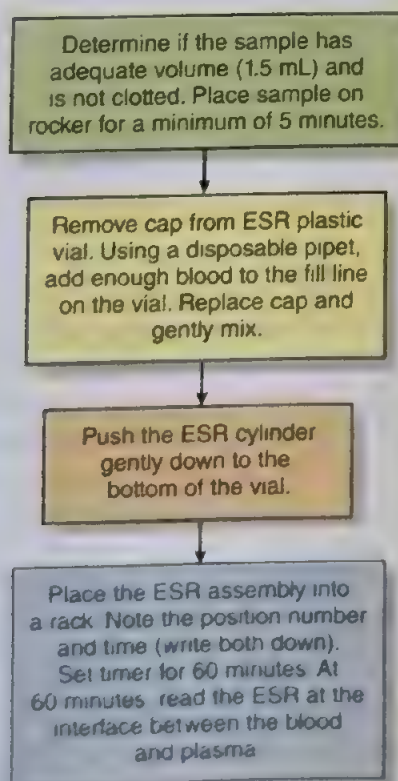


FIGURE 5-4 Flowchart of a sedimentation rate procedure.

and computer systems. Autoverification is an example of an automated examination system that contains set criteria for the release of test results determined by the manufacturer. The laboratory is responsible for verifying the release of test results by validating that the system has the capability to compare results with predetermined criteria and will not release results that have not met the criteria. When laboratory processes require change, a change management plan will provide justification and a plan for implementing the new process.

Documents and Records Management

The focus of this QSE is the creation, management, and retention of laboratory policies, procedures, forms, and records. There needs to be a process to identify and control documents as well as create, review, and approve new documents. The most important aspect of this QSE is ensuring the use of the current approved version, whether it is a laboratory policy, the testing procedure, or a form used for recording information.

Laboratories must comply with record retention requirements according to the rules of state and federal regulatory agencies.

Information Management

Multiple areas within the laboratory must be concerned with the control of information. Information management provides processes to access patient data, in paper and electronic form, to manage confidential information as well as internal and external threats. Determining appropriate access to patient information by employees, such as electronic password requirements and conducting audits for risk management, are within the scope of information management. Confidentiality, security, and data integrity are the foundation of information management.

Nonconforming Event Management

Nonconforming events (NCE), such as accidents, errors, and customer complaints, are any event that does not meet the specified requirements. NCEs require a process for detection, documentation, and analysis for identification of any trends. Laboratory leadership is responsible for creating and encouraging a culture to communicate these processes. The severity of the NCE will determine the action taken. A high-risk event may require an RCA or additional investigation to identify the cause to prevent or reduce the possibility of a reoccurrence.

Assessments

There are **internal assessments** and **external assessments**. Internal assessments include correlations between similar instruments, audits, self-inspections, and quality monitors. These assessments examine how the laboratory is meeting its own requirements. External assessments are activities involving outside sources. A few examples of external assessments are proficiency testing, regulatory surveys, and/or accreditation inspections. The purpose of these assessments is to evaluate the effectiveness of the laboratory processes.

Continual Improvement

Continual improvement is a proactive strategy to reduce risk and prevent nonconformances. Activities to monitor may be the result of assessment findings, customer complaints, and high-risk events. Quality indicators, which are determined by

laboratory leadership, are used to monitor performance and identify areas for improvement.

To summarize, the QMS becomes a circle of quality, with each QSE flowing into the next one (Fig. 5-5). Laboratory quality begins with the commitment of laboratory leadership, moves through the various QSEs, and finally, with continual improvement, circles back to the laboratory leadership to continue propagating quality into daily and future laboratory processes.

CRITICAL THINKING QUESTION

- 5-1** A friend learns that you work in a laboratory and asks, "Can you really trust the results that you get from lab work?" How can you answer them to communicate the level of quality assurance laboratory analysis contains?

See answers to all Critical Thinking Questions at the back of this book.

Quality Assurance and Quality Control

Key Definitions

The terms **quality assurance** and **quality control** are often used interchangeably to refer to ways of ensuring the quality of a service or product. However, these two terms have different meanings:

- **Quality assurance** is the planned and systematic activities implemented in a quality system so that quality requirements for a product or service will be fulfilled. Quality assurance is a direct result of performing quality assessments. If the proper method validation was

performed, quality requirements met, and assessments completed, the clinician is *assured* that the test results are of the highest quality possible.

- **Quality control** is the measurement of deviation from the average that is used in the interpretation of laboratory test results as well as monitoring the laboratory performance.
- **Quality assessment** is a quality effort that is concentrated on assessing the testing process, procedures, and corrective action to prevent future problems.

Important quality control definitions include:

- **Assayed control material** - A commercially prepared assayed control has expected mean and SD values provided by the manufacturer. The laboratory will need to validate the values, usually by running a new lot of QC before an old lot has been exhausted or expired, referred to as parallel testing, before running the new lot of control material to monitor patient samples.
- **Control chart** - A graphical method to display control results so that it is easier to determine whether a control run is in or out-of-control. Shifts and trends can be visually assessed using control charts.
- **Control limits** - Statistical criteria of acceptability for a particular control material, usually derived from the mean and SD. The limits are placed on a control chart for ease of use.
- **Control rule** - A decision criterion for judging a control material as acceptable or not. Westgard MultiRules are examples of control rules.

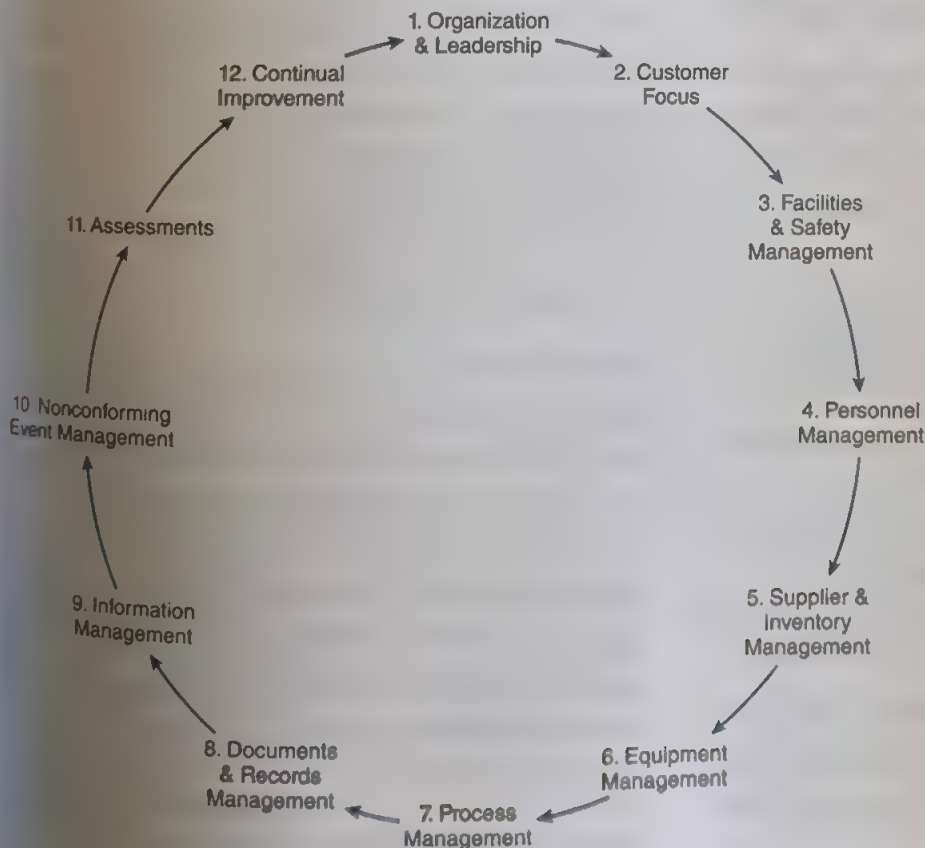


FIGURE 5-5 The Circle of Quality.

- **Delta checks** - Comparison of the difference between measurements of an analyte (or combinations of analytes) on two separate samples from the same patient to pre-defined thresholds representing the limits of acceptable change.
- **Matrix** - A term describing the substance of the QC material. Regulations require that the control material be as close as possible to real human specimens. For example, urine tests should be controlled by a urine-like control material, etc. Sometimes animal materials are used because they are very close in nature to the human materials and easier to manufacture.
- **Moving averages** - Researchers discovered that red blood cell indices (mean corpuscular volume [MCV], mean corpuscular hemoglobin [MCH], and mean corpuscular hemoglobin concentration [MCHC]) were stable in a normal person over a long period of time.¹³ The mean of the RBC indices in the population of a laboratory are monitored for significant drifts or shifts in calibration, which may mean method quality problems. This QC procedure should not be the only source of QC of RBC indices, and laboratories running fewer than 100 CBCs a day should not utilize this procedure due to lack of sufficient data.^{14,15}
- **Quality control levels** - Each control level should be carefully chosen to monitor a critical medical decision level whenever possible.
- **Quality control material** - The purpose of a QC material is to monitor the analytical quality of a method to alert the operator when there is a problem.¹² Most laboratories purchase commercially prepared materials due to ease of preparation, stability, and long shelf-life. At least two levels of controls are usually utilized per method.
- **Quality control statistics** - There are many mathematical calculations available to help determine whether a method is accurate and precise. Examples include Levy-Jennings plots, Youdin plots, Critical-Error graphs, Power Function graphs, correlation coefficient (r), linear regression, and t and F tests.
- **Run** - A finite length of time or number of patient samples. QC is designed to be analyzed at the beginning and end of a run. The CLIA definition of a run for QC purposes is 24 hours for most analytes. However, coagulation and a few other tests require QC analysis every 8 hours.
- **Shift** - An abrupt move in which six or more consecutive QC values are above or below the mean.
- **Statistical process control** - Statistical process control is a general term for the parts of a control system in which statistics are used, such as a Levy-Jennings graph.
- **Trend** - A slow change in QC values on a QC chart, either rising or falling steadily by a set of six or more consecutive data points.
- **Unassayed Control Material** - A commercially prepared control that does not have specific ranges verified by the manufacturer. The laboratory must run the control material in replicate over several days or weeks to establish an initial mean and SD.

General Quality Assurance Control Activity Guidelines

The CFR CLIA provides a broad outline of quality assurance activities based on a quality systems approach that follows the route of a specimen through the laboratory. The standards for quality systems are under the Analytic Systems section of subpart K³:

- Procedure manual (§493.1251)
- Test systems, equipment, instruments, reagents, materials, and supplies (§493.1252)
- Establishment and verification of method performance specifications (§493.1253)
- Equipment maintenance and function checks (§493.1254)
- Calibration and calibration verification procedures (§493.1255)
- Control procedures (§493.1256)
- Comparison of test results (§493.1281)
- Corrective actions (§493.1282)
- Test records (§493.1283)
- Analytic systems assessment (§493.1289)

Preanalytical, Analytical, and Postanalytical Factors in Testing

Common sources of laboratory errors can be categorized as preanalytical, analytical, and postanalytical (Table 5-1).

Preanalytical Factors

Preanalytical factors are the most common source of laboratory errors.¹¹ From the time the health-care provider orders the test through the processing and delivery of the specimen to the laboratory, each step along the way has the potential for error. Proper identification continues to be a concern, as is collection and labeling of the specimens in the presence of the patient. Bar-coded labels have improved this process over the years. Timing of specimen collection for specific tests is a challenge, and often the specimen is not collected at the exact time necessary for optimum quality results. Deviations from the specimen transport policies reduce the quality of the specimen and ultimately the quality of the result. Specimens that need to be analyzed within a short time frame, such as coagulation tests, should be tested as soon as possible or stored appropriately.

Analytical Factors

Analytical factors are errors that occur during the testing phase of a specimen. For both manual testing and automated instruments, possible errors may include imprecision and inaccuracy of the test method. Imprecision and inaccuracy can be the result of many factors including calibration drift, reagent issues, instrument malfunction, and specimen collection issues such as hemolysis or clotting. The QC procedures chosen should be the result of a logical evaluation of the method's errors, probability of error detection and false rejection, control rules, and number of control measurements. Testing personnel must be trained and competent to perform and report out the results.

Postanalytical Factors

Postanalytical factors are errors that occur after the test result has been released. Examples of these errors could be failure to

TABLE 5-1 Errors in the Testing Process

Phase of Testing	Common Errors
Preanalytical	<ul style="list-style-type: none"> • Patient identification • Sample identification • Sample transport • Sample handling
Analytical	<ul style="list-style-type: none"> • Calibration • Hemolyzed or Lipemic specimen • Quality control • Pipetting
Postanalytical	<ul style="list-style-type: none"> • Result entry • Calculations • Delay in issuing corrected report • Failure to communicate critical test result

communicate a critical value or a delay in issuing a corrected report, both of which could directly affect patient care and safety.

Accuracy, Precision, and Error

Accuracy describes a test result that is close to its true value, while **precision** refers to reproducibility (i.e., whether the same test can be run on the same sample and produce the same result). The target analogy is used in Figure 5-6 to visually describe accuracy and precision,¹¹ and Table 5-2 outlines the differences between the two concepts.

All analytical methods have some **error**. Error is the difference between the true value and the obtained value. How much accuracy and precision are necessary or desirable? What is the allowable error and bias for the method? These questions should be answered before purchasing a new instrument or performing a new analytical method.

How to Measure Accuracy

Accuracy is measured in **bias**, the difference between the mean of a set of replicate measurements and the true value. Accuracy may be expressed as a percentage or as a number with units. The **mean** (average) of a set of numbers shows **systematic error**, or error inherent in

Mean = the arithmetic average of a set of values –

$$\text{mean} = \frac{\sum x_i}{n} = \bar{x}$$

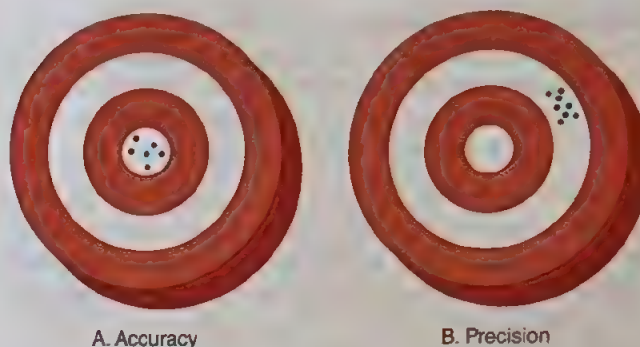
where n = number of observations (data points) and $\sum x$ = sum of all data

For example, if a sample were run 10 times for hemoglobin with the following results:

8.0, 8.5, 9.0, 7.8, 8.8, 9.1, 8.9, 9.1, 8.6, 8.8 = the mean is the average, which is 8.7. (To obtain the mean, the numbers are added together and then divided by 10, the number of observations.) How accurate is the data? The “true” value would need to be known. In this case, the true hemoglobin value was really 8.8. Calculate the accuracy two ways:

$$\text{Accuracy (bias)} = 8.8 - 8.7 = 0.1 \text{ g/dL or}$$

$$\text{Accuracy (bias)} = 0.1 / 8.8 \times 100 = 1.14\%$$



A. Accuracy

B. Precision

FIGURE 5-6 Accuracy and precision can be compared with a target.

A. Example of accuracy and precision (the shooter hit the “target” value, i.e., the bull’s eye, and also reproduced the shot several times). **B.** Precise, but not accurate.

TABLE 5-2 Differences Between Accuracy and Precision

Accuracy	Precision
Measured in bias	Measured in SD or CV
Measures systematic error	Measures random error

Accuracy studies show *constant systematic error* or *proportional systematic error* (both are biases). Accuracy studies should be performed with a patient sample repeated at least 10 times within a short period of time. How the constant and proportional systematic error relates to the mean is shown in Figure 5-7.

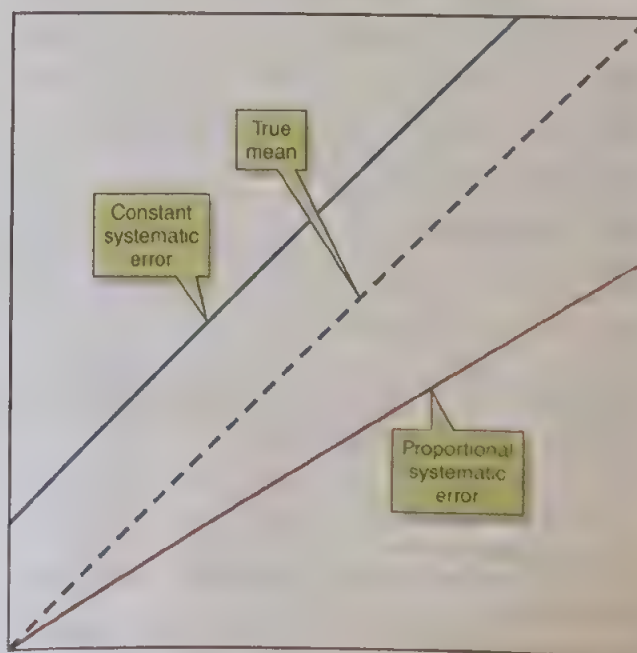


FIGURE 5-7 Accuracy: constant and proportional systematic error in relation to the mean.

How to Measure Precision

Precision is measured by the **standard deviation (SD)** or **coefficient of variation (CV)** of a set of replicate measurements (i.e., where did the five shots land in relation to the target?). Precision studies will show *random error*.

Standard Deviation Equation The standard deviation is determined by first calculating the mean, then taking the difference of each result from the mean, squaring that difference, dividing by $n - 1$, then taking the square root. All these operations are outlined in the following equation:

Standard deviation (SD or S):

$$S = \sqrt{\frac{\sum (x_i - \bar{x})^2}{(n - 1)}}$$

where s represents the standard deviation, Σ means summation of all the $(x_i - \bar{x})^2$ values, x_i is an individual result, \bar{x} is the mean of the results, and n is the total number of results included in the group.¹² For example, to calculate the SD, first observe a set of results and then calculate the mean of those results. For the hemoglobin study, the number of observations was 10, and the mean was 8.7. The calculated SD is 0.45.

The standard deviation is related to the spread or distribution of results about the mean. While the mean is an indicator of accuracy or systematic error, the standard deviation is a measure of the width of the distribution and is related to imprecision or random error. The bigger the standard deviation, the wider the distribution, the greater the random error, and the poorer the precision of the method; the smaller the standard deviation, the narrower and sharper the distribution, the smaller the random error, and the better the precision of the method.¹²

When using a measurement procedure, it is generally expected that a group of values will range from the smallest to the largest with most of the values clustered in the middle range. This is called a normal range or **Gaussian distribution**. The symmetry of the curve represents the central tendency where the central value in the normal distribution is the average or mean value. The percentage of results that are expected within certain limits (1, 2, and 3 SD) can be predicted. Approximately 68% of the values will fall within 1 SD, 96% of the values will fall within 2 SD, and 99% will fall within 3 SD (Fig. 5-8). When the values do not peak at the center, they are considered skewed.

Coefficient of Variation The coefficient of variation (CV) describes the standard deviation as a percentage of the mean, as shown in the following equation:

$$CV = (s / \bar{x}) 100$$

where s is the standard deviation, \bar{x} is the mean, and the multiplier of 100 is used to convert the s/\bar{x} ratio to a percentage.

For example, in the hemoglobin study the standard deviation (0.45) is divided by the mean (8.7) and multiplied by 100. The CV for the hemoglobin study is 5.2%.

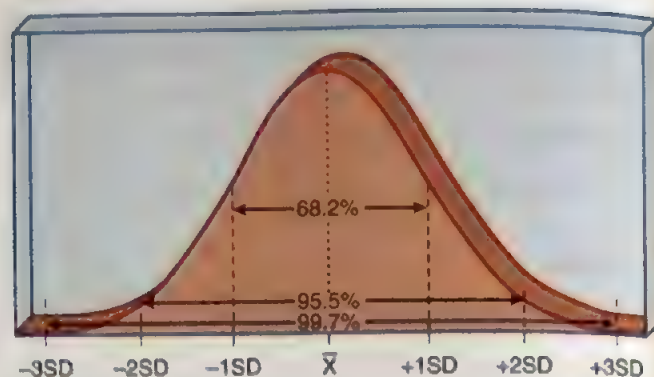


FIGURE 5-8 Example of Gaussian distribution curve for data of normal populations.

The coefficient of variation (CV) shows random error across a wide range of concentrations.

Since the % CV lacks units, the standard deviation among different assays, instrumentation, or laboratories can be compared by using the % CV. A set of measurements with a smaller % CV indicates the values are closer to the mean and has better reproducibility or precision of the test results.

Method Validation

How does a laboratory select a new method?¹² There are three general categories to consider:

- **Application characteristics** should include volume of specimen, type of specimen, workload appropriate for the testing situation (high-volume centralized lab vs. low-volume point-of-care), operator skills, cost per test, time for analysis, and operator training requirements.
- **Methodology characteristics** should include type of standards or calibrators, reagents and reaction conditions, traceability of standard or calibrator assigned values, method principle, measurement principle, and measurement capabilities.
- **Performance characteristics** should include working range, imprecision, interference, bias vs. the reference method, and total errors less than 10%.

When you are ready to begin the method validation process, a plan of action is very helpful (Box 5-1).

Studies to Introduce New Methods and Instruments

When a new instrument or method is introduced to the laboratory, a series of tests and calculations must be performed to validate each parameter.¹² The responsibility has been shifted to the laboratory to run the studies necessary to show that the method is accurate and precise. Even if the manufacturer has already performed some studies and has the printed data to support their claims, the laboratory must validate the studies required by CLIA.³

- Accuracy (bias)
- Precision (SD or CV)
- Reportable range of test results for the test system (linearity)
- Manufacturer's reference intervals (normal values): Are they appropriate for the laboratory's patient population?

BOX 5-1 Method Validation Plan

- Familiarization period
 - Establish working procedure.
 - Check working range.
 - Check calibration.
 - Check detection limit.
 - Preliminary MV experiments
 - Perform within-run replication study.
 - Perform interference study.
 - Perform recovery study.
 - Judge analytical acceptability.
 - Final MV experiments
 - Perform total replication study.
 - Perform comparison of methods study.
 - Judge analytical acceptability.
 - Verify reference interval(s).
 - Document studies.
 - Implementation
 - Select.
-
- Other studies that are usually performed: Comparison of Methods and Recovery experiments

Brief descriptions of the different method validations a laboratorian will need to run are provided in Box 5-2.

Interference Experiment

An **interference experiment** provides information about the constant systematic error (accuracy, bias) caused by interfering substances. Examples of interfering substances in hematology and coagulation are lipemia and hemolysis. For best results, obtain fresh lipemic patient samples; alternately, a commercial lipid emulsion can be purchased. Several concentrations of lipemia are created in a linear fashion by adding different amounts of the lipid to a normal whole blood specimen. At least three different specimens should be used for the experiment to decrease the random error. The test samples are analyzed in duplicate by the test method to determine the point at which the results are valid (within a predetermined percentage of the original normal result). Hemolysis can be induced by freezing and thawing aliquots of patient CBCs. Test samples are prepared as in the lipemia experiment. The average result of each duplicate pair is calculated, and then the difference between the total average normal values and the total average altered values is determined. This difference

is compared with the CLIA standards for total allowable error (TE). If the experiments revealed an interference difference greater than the CLIA standards, then the method or instrument would not be acceptable.

Replication Experiment

A **replication experiment** provides information about *random error* (precision, SD, or CV) and is performed by making replicate measurements on a series of patient samples and/or QC material within a specified period of time, usually within an analytical run, within a day, or over a period of a month. The preliminary experiment usually involves determining within-run imprecision by running a sample 10 or more times within one run. The final experiment generally requires at least 20 working days to provide a good estimate of the total imprecision, which includes within and between run components.

Reportable Range (Linearity) Experiment

A **reportable range (linearity) experiment** is useful to assess the lowest and highest values that can be reliably reported. It is good laboratory practice to only report values that can be proven accurate and not rely on manufacturer's claims. Linearity kits can be purchased for most hematology analytes that contain five or more levels for testing. Each level is tested at least three times. The statistical charts and graphs needed to make decisions are usually provided by the manufacturer of the linearity kit. The laboratory establishes expected limits for each parameter at each level. A best-fit linear line is drawn through all the points on a plot of the means of the measured values (y-axis) vs. known values (x-axis). The low and high ends of the reportable range are the values just before the laboratory-established limits are exceeded.

Reference Range (Normal Range) Experiment

A **reference range (normal range) experiment** is essential to establish a new reference range or to verify the manufacturer's normal range claims. An experiment to establish a new range could include more than 120 specimens per age and sex, if the ranges changed significantly across these categories. If the new method has well-documented studies available either from the manufacturer, in literature, or in-house, then a smaller study of 20 samples may be useful to validate the range: if two samples or fewer fall outside the stated ranges, then the ranges are statistically verified. If there is any question as to the validity of the original study to be transferred or if the new method is significantly different from the old method, then a larger validation study of at least 60 samples should be performed.¹³

Comparison of Methods Experiment

A **comparison of methods experiment** is primarily used to estimate the average systematic error observed between two similar methods but can also reveal the constant or proportional nature of that error. The results of patient specimens are compared to determine the analytical errors between the methods. A minimum of 40 well-chosen patient samples should be tested over a minimum of 5 working days. These samples should be distributed one-third in the low to low-normal range, one-third in the normal range, and one-third in the

BOX 5-2 Method Validation Experiments

- Replication experiment: within-run and between-run for random error
- Interference experiment: make dilutions with the interfering substance for constant systematic error
- Recovery experiment: make dilutions of patient samples for proportional systematic error
- Comparison of Methods experiment: run patient samples on two test methods for average systematic error

high abnormal range. The correlation coefficient (r) is a useful statistical tool for comparing two sets of data. A perfect linear relationship between the data would result in a correlation coefficient of 1.0. Figure 5-9 is an example of a correlation study between identical hematology analyzers. Each lab must establish its own correlation acceptable limits for each assay. **Regression statistics** are another mathematical comparison of two sets of data; systematic differences can be detected more readily with this method. Once the data have been collected, method acceptability should be judged based on the sizes of the random, systematic, and total analytical errors. If these errors are small compared with the amount of error that would invalidate the use and interpretation of a test result, the method is acceptable. If the errors are too large, it may be necessary to reject the method or to identify and eliminate the causes of the errors. The laboratory director should be consulted when the data are difficult to interpret.

Recovery Experiment

A **recovery experiment** can be used when there is no comparison method. The purpose is to uncover proportional systematic error. Commercial solutions of high concentrations of the analyte to be measured are obtained and added in a linear fashion to patient samples, and the samples are run two or more times to reduce the random error. The results are averaged and compared, and a percent difference is calculated. The total error defined by CLIA is compared with the percent difference: if the difference is greater than the total error allowed, the method or test is not acceptable.

CRITICAL THINKING QUESTION

5-2 Random error is a realistic part of everyday laboratory analysis and should be recognized and addressed. How do interference and replication experiments introduce enough error to provide quality assurance for random errors?

Delta Checks

Delta checks are used for internal quality assurance and to ensure compliance with the CLIA requirement that laboratories have a system to identify and assess patient test results that appear inconsistent.¹⁶ The delta check concept was developed in 1967. However, it wasn't widely implemented until the early 1970s with the development of laboratory information systems (LIS).^{17,18} Delta checks detect significant changes in sequential patient test results. These changes can indicate a critical change in a patient's condition, such as an unexpected drop in hemoglobin, or a possible misidentification, which is a major patient safety event. Errors may also be related to improper specimen collection such as hemolysis or specimens that are lipemic or clotted.

Recall that delta checks compare the difference between measurements of an analyte (or combinations of analytes) on two separate samples from the same patient to predefined thresholds that represent the limits of acceptable change. Delta checks are used to assess specimen integrity and detect errors before the release of results into the patient record.¹⁹ They can also indicate a significant change in a patient's status and

LH1 HGB	LH2 HGB
8.8	8.8
9.2	9.2
9.3	9.2
9.4	9.2
9.6	9.6
9.6	9.5
9.9	10.1
10.1	10.1
10.5	10.4
10.6	10.7
10.7	10.6
11.0	11.0
11.3	11.2
11.3	11.3
11.4	11.5
11.6	11.5
11.7	11.6
11.7	11.7
12.1	11.9
12.2	12.1
12.4	12.2
12.5	12.4
12.7	12.8
12.7	12.7
13.1	13.0
13.2	13.1
13.6	13.5
13.7	13.6
13.8	13.6
13.9	13.8
14.2	14.1
14.7	14.7
15.0	15.0

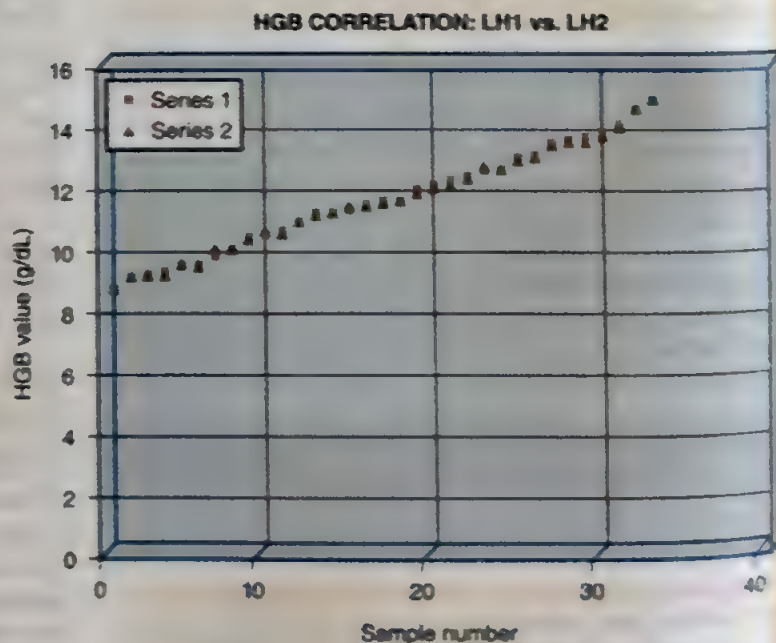


FIGURE 5-9 Correlation study of hematology analyzers (hemoglobin).

provide critical information for the clinician. The menu of analytes is usually tests which have low physiological variation and are repeated frequently during short periods of time. The most common analytes are electrolytes, hematology tests (hemoglobin, hematocrit, platelet count, white blood cell count, mean corpuscular volume, red blood cell distribution width), blood urea nitrogen, and creatinine. The preferences for the delta check guidelines should be tailored for the individual laboratory. The median time interval set for calculating delta check ranges depends on the patient population, the capacity of the LIS, and the oversight of the medical director.

When a delta check alert occurs, a thorough investigation should be initiated. Use of a standardized procedure or a flowchart is both beneficial and recommended for consistency in documenting the investigation. A College of American Pathologist (CAP) Q-Probe Study involving 49 health-care facilities and 6,541 delta check alerts found that almost all testing problems involved specimen collection issues such as hemolysis, contamination with intravenous fluids, or clotting. The frequency of analytic errors detected by delta check alerts was 1.1 per 1,000 testing episodes.²⁰ In the event of a suspected mislabeled specimen, repeat testing of both the current specimen and previous specimen should be performed when possible.

The most common problem associated with delta checks are a change in the patient's condition. When this occurs, the action taken involves a review of the patient's medical record. In this situation, the delta check is not considered an error.²¹ An example of this type of alert would be a presurgical patient with a hemoglobin value of 7.1 g/dL at the time of admission who had surgery and a hemoglobin of 9.3 g/dL the following day. The delta alert would flag the patient's result because of the change in hemoglobin within 24 hours. The next step would be to review the patient's medical record, revealing the patient had received 2 units of packed red blood cells during surgery. A comment appended to the result stating the patient had received a red blood cell transfusion would explain the increase in hemoglobin.

In computerized laboratories, to reduce test turnaround time (TAT) and improve efficiency, many clinical laboratories have adopted delta checks as a critical component of the autoverification practice. By having well-developed criteria for acceptance such as defined critical values and the ability to detect icteric, lipemic, or hemolysis in blood specimens, the workflow and error detection technology have improved allowing more results to pass.

CRITICAL THINKING QUESTION

5-3 How do delta checks play a critical role in quality control?

CLIA Minimum Quality Control Requirements

The CLIA requirements specify:

- Run two levels of controls for quantitative tests each day when patient specimens are assayed. Manual hematology cell counts require one control every 8 hours, nonmanual

coagulation requires two levels of control every 8 hours, but manual coagulation tests require two levels of control each time specimens are analyzed.

- Test controls after a complete change of reagents, preventive maintenance, or replacement of a critical part or component.
- Rotate controls among all operators performing the test.
- Test control materials the same as patient materials.
- Establish or verify criteria for acceptability for all control materials.
- Determine the statistical parameters (mean and SD) over time through concurrent testing for all unassayed control materials.
- Make sure controls meet criteria before reporting patient test results.
- Document all control procedures performed (Box 5-3).
- All new QC lots must be run in parallel with current control material to establish or verify the acceptable range before using the new control lot to monitor patient results. The number of data points needed is dependent on several factors such as the stability of the test system, the stability of the control material, and laboratory policy. For automated hematology analyzers, a parallel experiment of a few runs per day for several days on all shifts should be sufficient because most systems and the commercially prepared control material are very stable. For new lots of coagulation controls, a few runs per day for a week or more may be necessary depending on whether the controls are assayed (fewer runs to validate mean) or unassayed (more runs to establish mean).

Almost all current regulations require a laboratorian to establish an in-house mean and SD for any QC material to be used before the material is placed in service to monitor a method. Why is this necessary? Even if the manufacturer supplies ranges and SDs, the data are usually combined from many instruments and possibly multiple methods to arrive at the acceptable mean and SD listed in their package insert, which usually makes the ranges wider than is useful for daily method monitoring. In addition, the manufacturer's data cannot predict how the control material will behave on a particular lab's instrument as handled and run by the staff. Coagulation laboratories have been establishing control means and ranges for years with unassayed materials and for special tests that require frequent calibration.

BOX 5-3 CLIA Minimum QC Requirements

- Run two controls each day of testing for quantitative tests.
- Run controls after changing all reagents, preventive maintenance, or replacement of a critical part or component.
- Rotate QC among the staff.
- Test QC the same as patients.
- Establish or verify the QC ranges.
- Establish a mean and SD for new unassayed QC before going live.
- Do not report patients unless QC is within limits.
- Document all QC procedures performed.

Hematology is somewhat trickier: the commercial QC lot number changes every 6 weeks or so. Calculating new SDs every 6 weeks is not very useful or productive. To establish in-house ranges, a lab scientist could average the SDs for each CBC and differential parameter from all control levels from the instrument peer group reports for the past 6 months. The average SDs are used as the basis for calculating in-house ranges. The controls are run and means established for each parameter. The instrument software should allow the user to choose these new means as the target means. New ranges are then calculated while ensuring that the new ranges are not wider than the manufacturer's package insert ranges. The SDs can be entered into the instrument for future use with each new lot of controls. To test the new ranges, run the controls a few times every day for a week. If the control values fall outside the new ranges, then the new ranges may be too "tight" and need to be widened a bit. Multiply the SD by 2, then 3, then 4, and so on until the controls are within the new range. Document the process adequately for inspection purposes. Note: some differential parameters, such as basophils and eosinophils, normally have a high SD due to relatively low actual values.

Levy-Jennings Graphs

Levy and Jennings introduced the idea of control graphs in 1950.²² The **Levy-Jennings (L-J) graph** used today consists of individual data points plotted on a graph of the mean and the control limits of individual control levels. The L-J graph historically has consisted of 1, 2, or 3 SD ranges. The roots of the 2 SD phenomenon can be traced back to Carl Frederick Gauss (1777–1855), who introduced the concept of the normal curve. Because 95% of the points in a normal data set for a particular parameter are expected to be within 2 SD of the mean, it seemed appropriate to use ± 2 SD limits for laboratory QC. Therefore, when Levy and Jennings started using a chart for QC, the 2 SD range was applied and has remained ever since. However, laboratories must establish their own mean and limits for each assay based on tests run in-house and calculations for quality (accuracy and precision). Figure 5-10 is an example of an L-J graph for WBC QC normal level.

The L-J graph is a visual view of how the QC is performing, and therefore, how the method is performing. According to the Gaussian distribution, data points should occur equally around the mean, which is true for most analytes. A *shift* in the values represents a sudden and consistent bias from the mean (i.e., a group of data points are clustered on one side of

the mean). A shift is representative of systematic error. It is commonly accepted that six points on one side of the mean constitutes a shift; however, every lab should determine how many data points constitute a shift. Causes of a shift include improperly prepared reagents or controls, reagent deterioration, change in lot numbers, and failure of an instrument component (Fig. 5-11).

A *trend* is a gradual change in the same direction, either increasing or decreasing (Fig. 5-12). A trend is also systematic error. Again, each lab must determine how many data points constitute a trend. Trends can be caused by the same items as a shift, but because the data are slowly changing, the root cause may be more difficult to identify. Look for items that can change over time, such as reagent or lamp deterioration, temperature fluctuation, calibration shift, and so forth. Use a systematic logical troubleshooting approach in isolating the cause, making only one change at a time, and documenting each action taken.

Another type of change commonly seen on an L-J graph is an increase in imprecision. The data points become further away from the mean, and the SD becomes larger. If there is contamination of the diluent water used in coagulation reconstitution, the QC material may display such a dispersion of results. Other causes of imprecision include poor mixing of the control material, pipetting errors, and so forth.

Westgard MultiRule Quality Control

When QC data points have been run and entered onto an L-J QC chart, the results must be reviewed. **Westgard Rules** is a series of decision criteria (control rules) to decide whether an analytical run is in-range or out-of-range. There are rules for two levels or three levels of control material. To improve sensitivity, it is suggested that at least two rules be used together for every control material level. Here is a list of the most used Westgard Rules:¹¹

- **1_{2s}** This rule refers to one data point exceeding the ± 2 SD limit.
- **1_{2.5s}** This rule refers to one data point exceeding the ± 2.5 SD limit.
- **1_{3s}** This rule refers to one data point exceeding the ± 3 SD limit.
- **2_{2s}** This rule refers to two consecutive data points exceeding the ± 2 SD limit.
- **R_{4s}** This rule refers to one data point exceeding the ± 2 SD limit, and another close data point exceeds the -2 SD limit

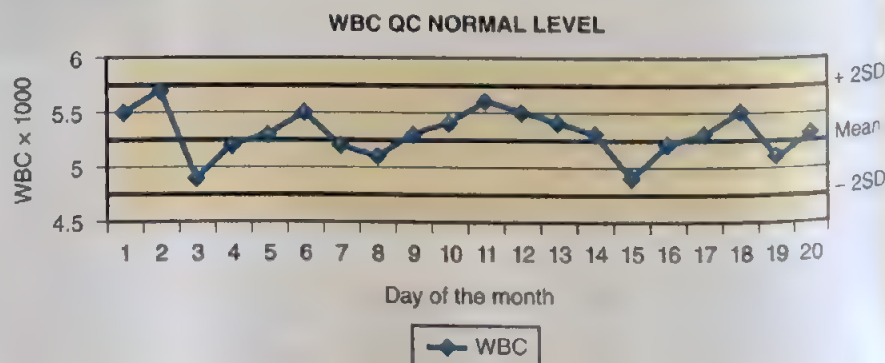


FIGURE 5-10 A typical Levy-Jennings graph. Each data point is plotted so an overall view of the data is possible.

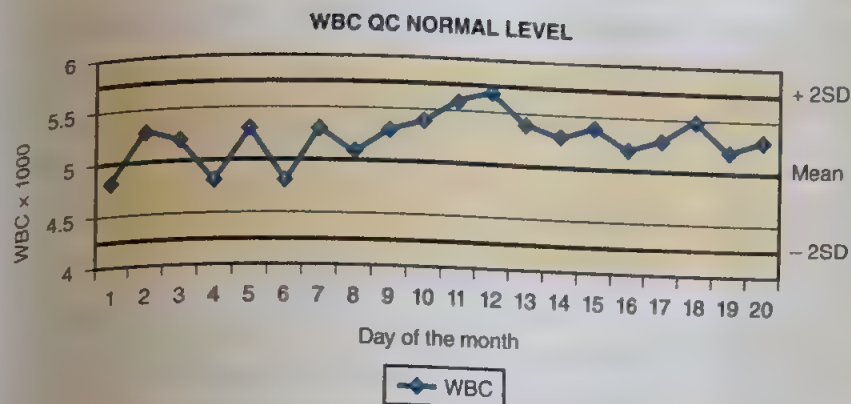


FIGURE 5-11 Example of a shift in QC values on a Levy-Jennings graph. Notice that between day 8 and day 20 the data has abruptly shifted above the mean.

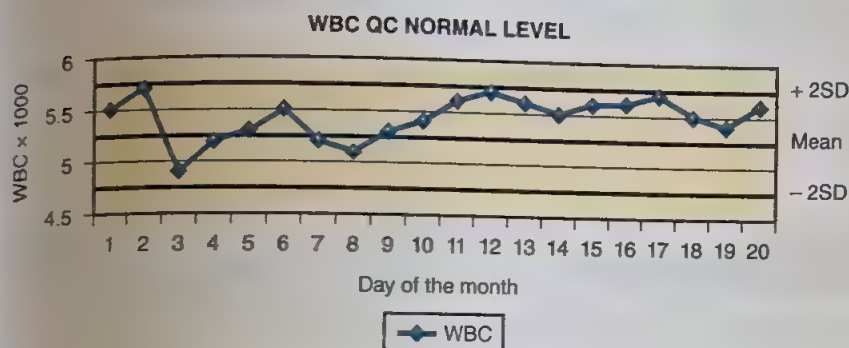


FIGURE 5-12 Example of a trend in QC values on a Levy-Jennings graph. Notice that between day 8 and day 20 there is a slow trend above the mean.

(opposite directions). An example of this rule is illustrated in Figure 5-13.

- **2 of 3_s**. This rule refers to two out of three control points exceeding either the +2 SD or the -2 SD limit on the same side.
- **7_T**. This rule refers to seven consecutive data points trending in the same direction, either progressively lower or higher.
- **4_s**. This rule refers to four consecutive control measurements exceeding the +1 SD or -1 SD limit on the same side of the graph.
- **10_s**. This rule refers to 10 consecutive control measurements falling on one side of the mean.

Systematic Errors

If a multitest system is being run (i.e., automated CBC), how many of the parameters were out of range? If more than one parameter was affected, then the troubleshooting should be

logically aimed at processes that the parameters have in common. For example, the WBC counting chamber also determines the Hgb parameter. Another route to investigate is any recent changes to the system, reagents, calibration, and maintenance, as they cause many systematic errors.

Random Errors

The first and easiest way to troubleshoot random errors is to inspect the system while it is operating. Watch every process that can be visually accessed. Look for loose seals, bubbles, leaks, faulty delivery volumes, clogs, and any mechanical parts and reagents. If there is nothing visually wrong, consult the operator's manual and troubleshooting guide for assistance. Ask key operators to review the error log for clues. Call Technical Service for suggestions or have an engineer inspect the instrument. Run a precision check of at least 10 replicates of a fresh patient sample to rule out imprecision. If no problems can be detected, add another random error

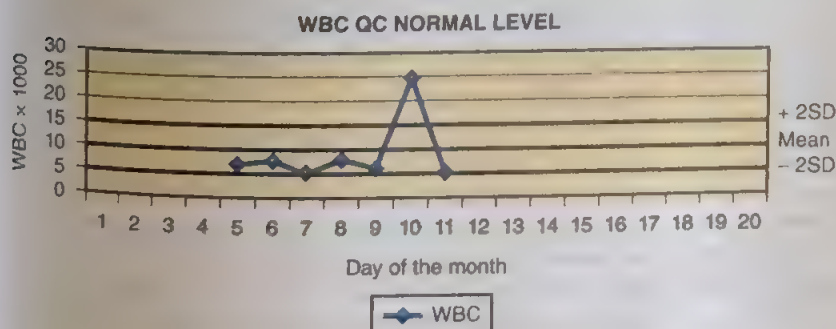


FIGURE 5-13 A Levy-Jennings graph showing a violation of the Westgard Rule R_{4s} .

Westgard Rule to help detect a future error and monitor the system (tell all staff about the problem also). Always document all investigations and findings in a problem log for the method or instrument.

Peer Group Quality Control

Most of the larger companies that manufacture instruments and/or QC products have a peer group QC program. The daily or monthly data are sent to the company to compare with other labs running the same instrument or QC material for the same assays. This serves two purposes: to satisfy the CLIA requirements for outside assessments and to provide feedback about a laboratory's methods versus peers. Review the grouping criteria to be sure that the instrument is in the correct group according to reagent lot number or control lot number. Knowing how a method compares with peers gives laboratorians one more tool in their quality toolbox.

Statistics

The manufacturer will probably provide peer statistics in their report containing one or more of the following:

SDI (Standard Deviation Index): SDI compares your mean to the group mean. This calculation describes the systematic error or bias of a method as a multiplier of the SD for the group:

$$\text{SDI} = (\text{Lab mean} - \text{Group mean}) / \text{Group SD}$$

An SDI of 0.0 indicates that the lab mean is the same as the group mean. A SDI of 2.0 or higher requires investigation: the method is in danger of systematic inaccuracy. An advantage to using SDI is that a person can scan a peer group report and view the SDI of all the assays fairly quickly.

CVI (CV Index): The CV of the lab and the CV of the group compared:

$$\text{CVI} = \text{Lab CV} / \text{Group CV}$$

A CVI of 0.0 indicates the laboratory's CV is the same as the group CV. Any CVI greater than 0.0 suggests that the laboratory's imprecision is less than that observed for the group. A CVI greater than 1.0 suggests the lab's imprecision is higher than that of the group.

Z-score: Measures how many standard deviations the mean of an analyte is from the mean of the peer group. A Z-score has no units; however, the score can be positive or negative.

$$\text{Z-Score} = \text{analyte mean score} - \text{the Peer mean} / \text{Peer SD}$$

Review of Peer Group Data

While reviewing peer data, the laboratory's assay mean should be compared with the peer's mean. If the lab's mean is consistently above or below the peer mean each month but the lab's SDI and CVI are acceptable, it may be time to consider an adjustment in the ranges. QC ranges need a little adjustment now and then as part of the normal instrument and QC material process. A good rule of thumb is to adjust QC ranges only every 90 days and less if possible. If the ranges were adjusted every month to match the peer mean,

the QC process would be negated in favor of looking good on paper.

As with any quality review, written documentation must be noted on the peer group report that it was reviewed and if any problems were seen with the data. If an assay requires investigation, the details should be written as if an inspector were reading them. Always date any reviews and list any corrective or preventive measures.

When implementing automation in the hematology laboratory, CLSI has published an approved standard *The Validation, Verification, and Quality Assurance of Automated Hematology Analyzers*.²³ This standard provides guidelines for instrument calibration and assessment of performance criteria, including accuracy, precision, linearity, sensitivity, and specificity. This standard is an excellent resource for assisting with critical evaluation and implementation of hematology analyzers.

By creating a quality culture within the laboratory, competency of laboratory staff, performance of quality testing, and release of accurate results can be built into the workflow. Following established quality assurance policies and procedures becomes less of a hardship. Competency programs verify all testing personnel are performing assays and reporting results properly, and quality assurance policies ensure instruments are releasing accurate and precise results, both of which result in better patient management and care.

Hematology Laboratory Applications

Quality Plan Example

Laboratory Alpha decides to combine selections from several Quality Management approaches to form their own unique Quality Plan. From the Lean approach, the staff follows a CBC, a PT, and a urinalysis sample from collection through result reporting to the physician. Samples are selected from different areas, such as outpatients, inpatients, and clinics to cover as many scenarios as possible. The staff monitors the motions of the sample and the employees to eliminate extra waiting, extra steps, and unnecessary movement. Any defects or extra processing steps are noted. The laboratory uses Lean techniques to remove the unnecessary steps and monitors the process for improvement. The laboratory must define how often the study will be performed, how often it will be collected and presented, and ongoing review and corrective actions. Many quality entities are covered in the plan, making it comprehensive. However, this would be a portion of a total Quality Management Plan. The leadership then decides to use Six Sigma tools for the processes. Once a goal is set, the team can institute a six-sigma tool to reduce the variation in the automated process, and improve the process.

Method Validation Studies

Once a new method or new instrument has been installed, method validation studies must be performed. The focus is to introduce about the same amount of variation that would be present daily in the normal running of the instrument. If the test will be performed on three shifts, validation samples should be performed on three shifts. The scientist who will be performing the test should analyze

of the validation samples. For normal patient studies, the goal is to duplicate the same types of patients tested every day. A plan is necessary to organize the samples needed for each study, the time required for each study, and the personnel who will be conducting the studies.

Hematology Laboratory Alpha has just installed a new automated hematology instrument. After the basic functions of the instrument are verified by the manufacturer, the validation studies are performed. A replication experiment is planned in two phases, preliminary and comprehensive. The preliminary phase is completed in 1 day with one run of 20 replicates of the same sample (within-run random error). The comprehensive phase includes analyzing quality control material of different levels on all shifts by several lab scientists for at least 20 days (between-run random error). The mean, SD, and CV are calculated for all parameters for both phases and are acceptable. A reportable range experiment is performed with a commercial CBC and reticulocyte linearity kit that covers as much of the analytical range as possible (proportional systematic error

and linearity). The manufacturer of the kit receives the data and calculates the statistics; the laboratory personnel review the data and decide on a reportable range. Because the new automated analyzer is similar to the current one, a small normal range study is performed with 20 carefully screened adult patient samples from both sexes, and the previous range is verified. For pediatric normal values, a decision is made to transfer the ranges by calculation from the pediatric sample portion of the comparison of methods study. The comparison of methods experiment is performed with 40 adult and 40 pediatric patient samples using normal and abnormal patients across the entire analytical range. The results of all the studies are acceptable.

Quality Control

Once the instrument performance has been investigated and deemed acceptable, the monitoring of the quality required needs to be planned and executed. For a hematology analyzer with multiple parameters, a critical parameter might be chosen, or a parameter with the highest SD; the laboratory must decide this.

SUMMARY CHART

- Quality Management comprises all the processes that affect the quality of a laboratory test results. Processes are designed with quality built in at each step supporting a culture of patient safety.
- CMS regulates all diagnostic laboratory testing performed on humans in the United States through the Clinical Laboratory Improvement Amendments (CLIA). CMS has deemed various accreditation organizations the ability to inspect and accredit clinical laboratories.
- Quality approaches include benchmarking, RCA, Lean, Six Sigma, FMEA, and DMAIC.
- 12 Quality System Essentials, created by CLSI, create the foundation of an effective, well-organized Quality Management System. Each QSE has a specific role in supporting the QMS: leadership/organization, customer focus, safety, personnel, supplier, equipment, process management, documents and records, information management, nonconforming event management, assessments, and continual improvement.
- A Quality Circle is created by the implementation of the 12 QSEs. The processes sequence begins with Leadership and goes full circle through all the QSEs, returning to Leadership. This system demonstrates the continual improvement cycle of ongoing quality analysis and assessment.
- Quality begins with the correct laboratory test order, specimen collection through the analysis, and reporting of the result. This is referred to as Preanalytical, Analytical, and Postanalytical Path of Workflow. Patient outcomes are considered part of postanalytical path.
- CLIA provides a broad outline of quality assurance activities which follow the patient specimen through the laboratory.
- Accuracy is measure in bias which is the difference between the mean of a set of values and the true value. Precision is measured by the standard deviation or coefficient of variation of a set of values.
- CLIA requires laboratories to perform method validation studies for all nonwaived methods. These studies include interference, replication, linearity, reference range, and comparison of methods.
- Delta checks are used for internal quality assurance and to demonstrate compliance with the CLIA requirement that laboratories have a system to identify and assess inconsistent patient test results.
- Levy-Jennings Graphs are used to graph QC data points to establish control limits. Westgard Rules consist of a series of decision criteria to determine whether an analytic run is in-range or out-of-range.

CASE STUDY 5-1

A medical laboratory scientist just ran three levels of controls on a hematology analyzer. The low level for WBC has been within 1 SD of the mean for the past 3 weeks; however, today the value is greater than 4 SD of the mean. The lab uses the following Westgard Rules: R_{4s} , $2\sigma 3_{2s}$, 7_T , 10_x .

QUESTIONS

1. What should be done next?
2. Is this a random or systematic error?
3. Do all the patients need to be rerun since the last low level was within limits?

ANSWERS

1. Most control material for hematology analyzers is packaged as a set of three levels: low, normal, and high. The Westgard Rule of R_{4s} has been violated; action is required. Errors from improper mixing of the control material should be ruled out first; the control is then rerun once. If the WBC low level is still outside 4 SD, a new control vial is opened, mixed, and rerun. A background check is also appropriate for troubleshooting problems with low levels of controls. If the background check is acceptable and the control is still outside 4 SD, observe the instrument during all phases of testing: are there any leaks, partial aspiration errors, interference flags, bubbles in any of the lines, buildup on the sampling valve? Zapping or bleaching the apertures may resolve the problem. Run a 10-sample precision check of a fresh patient or control. Review all the quality control on the instrument for the past few weeks for shifts and trends. Call Technical Service or check the operator's manual for more suggestions. If no source of the problem is found, add another Westgard Rule to detect future errors. At some point in the future, the additional rule may seem unnecessary and can be removed.
2. This is a random error. Only one parameter is affected. No shifts or trends were found when the QC was reviewed.
3. If the normal and high levels controls are within 1 SD, there may be an issue with the low range of reportable results. After the instrument is given a clean bill of health by an engineer, calibration and linearity studies may be appropriate to verify the reportable range.

CASE STUDY 5-2

A medical laboratory scientist is running 10 runs of a new lot of hematology control material with three levels: low, normal, and high. She notices that the low control has a much higher CV for the platelet count and the WBC than the previous lot number.

QUESTIONS

1. Is this a systematic or random error?
2. What are the next steps?
3. Is the problem more likely to be instrument/reagent-related or quality control material-related (manufacturing, handling)?

ANSWERS

1. This is a systematic error, affecting more than one parameter.

2. Handling of the control material should be ruled out first. All the QC records on this analyzer for the past

- few weeks should be reviewed for shifts and trends. Is there another identical or very similar instrument that uses the same controls? Compare how the controls are running on both instruments. Since the WBC and platelets are counted in the same chamber, look for instrument problems that affect this pathway. Check the background counts, check reagents and tubing, clean the sampling valve, bleach the apertures, etc. Observe the instrument as it processes a sample for any clues: bubbles, leaks, etc. The lower levels of controls may pick up a blockage or small leak first.
3. This is more likely to be an instrument problem. A high CV is hardly seen with mishandled control material; the cells are stabilized and are resistant to most mishandling.

REVIEW QUESTIONS

1. Which of the following organizations have been deemed by CMS to inspect and accredit the clinical laboratory?
 - a. American Society for Clinical Pathologists
 - b. College of American Pathologists
 - c. Clinical Laboratory Standards Institute
 - d. American Society of Clinical Laboratory Science
2. Which quality approach identifies nonvalue-added activities to streamline and standardize processes?
 - a. Six Sigma
 - b. Root Cause Analysis
 - c. Lean
 - d. Benchmarking
3. Which of the following examples would fulfil the minimal regulatory requirement to establish competency for testing personnel?
 - a. Direct observation of patient testing, review of worksheets, observation of equipment maintenance, ensuring problem-solving skills
 - b. Direct observation of patient testing, monitoring of reporting of test results, review of QC records, observation of equipment maintenance
 - c. Direct observation of patient testing, monitoring of reporting of test results, review of worksheets, ensuring problem-solving skills
 - d. Direct observation of patient testing, monitoring of reporting of test results, review of PT results, observation of equipment maintenance, ensuring problem-solving skills, assessment with blind testing performance
4. Which QSE is a proactive strategy to reduce risk and prevent nonconformances?
 - a. QSE Continual Improvement Management
 - b. QSE Assessment Management
 - c. QSE Nonconforming Event Management
 - d. QSE Process Management
5. An example of an internal assessment is:
 - a. Audit of unacceptable blood samples
 - b. Performing routine proficiency testing
 - c. Participating in an accreditation inspection
 - d. Performing an accreditation inspection
6. Which experiment provides information about random error?
 - a. Interference experiment
 - b. Replication experiment
 - c. Linearity experiment
 - d. Reference range experiment
7. For most analytes, the CLIA definition of a run for QC purposes is:
 - a. 4 hours
 - b. 8 hours
 - c. 24 hours
 - d. 48 hours
8. Which of the following is most commonly used for a delta check?
 - a. Hemoglobin and hematocrit
 - b. Plasma hemoglobin
 - c. Reticulocyte count
 - d. Prothrombin time
9. The Westgard MultiRule of R_{4s} indicates:
 - a. One data point exceeding the ± 2 SD and one data point exceeding the -2 SD
 - b. Two data points exceeding the ± 3 SD and one data point exceeding the -2 SD
 - c. Four data points exceeding the ± 1 SD
 - d. One data point exceeding the $+2$ SD and another data point exceeding the -2 SD
10. A slow rise in QC values on a L-J chart, steadily increasing by a set of six data points is a:
 - a. Shift
 - b. Trend
 - c. Run
 - d. Swing
11. The Lean improvement tool identifies seven types of:
 - a. Waste
 - b. Variables
 - c. Controls
 - d. Procedures
12. Which term indicates how close a result is to the true value?
 - a. Precision
 - b. Error
 - c. Accuracy
 - d. Shift
13. Laboratory staff take 10 extra steps to reach the workstation required for the second half of an analytic procedure. This is an example of which type of waste, according to the Lean tool?
 - a. Defects
 - b. Inventory
 - c. Waiting
 - d. Motion

Continued

REVIEW QUESTIONS—cont'd

14. The path of workflow considers variables for which stage of analysis?
 - a. Procedural stage
 - b. Preexamination stage
 - c. Flow stage
 - d. Inventory stage
15. Quality control differs from quality assurance in that:
 - a. QC is the planned and systematic activities implemented to fulfil quality.
 - b. QC is the assurance that test results are of a high quality.
 - c. QC is the measurement used to interpret laboratory performance.
 - d. QC is a direct result of quality assessment.
16. The most common source of laboratory errors is found in which stage?
 - a. Preexamination stage
 - b. Examination stage
 - c. Postexamination stage
 - d. Quality control stage
17. The spread of results about the mean is measured by which statistic?
 - a. Standard deviation
 - b. Median
 - c. Coefficient of variation
 - d. Mean
18. Which of the following is the central value in the Gaussian distribution?
 - a. The standard deviation
 - b. The mean
 - c. The coefficient of variation
 - d. The number of data points minus 1
19. Which of the following is a commercially prepared control substance with an expected mean and standard deviation?
 - a. An assayed control material
 - b. A peer control material
 - c. An unassayed control material
 - d. A delta check
20. Introducing error into a new method or instrument is part of:
 - a. Delta checks
 - b. Validation studies
 - c. The Lean process
 - d. Assessment of a Levy–Jennings graph.

See answers at the back of this book.

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CHAPTER 6

Anemia

Diagnosis and Clinical Considerations

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CHAPTER OUTLINE

Causes, Considerations, and
Compensatory Mechanisms
Clinical Diagnosis of Anemia
Classification of Anemia
Laboratory Classification of Anemias

Hemoglobin and Hematocrit Levels
Morphological Classification of Anemia
Other Laboratory Tests
New RBC Parameters in Testing for
Anemia

Overview of the Treatment of Anemias
Summary Chart
Review Questions
References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 6-1 List the causes of anemia and its common clinical signs.
- 6-2 Explain compensatory mechanisms in anemia.
- 6-3 State the laboratory criteria for the diagnosis of anemia.
- 6-4 Differentiate between etiological and morphological classifications of anemia.
- 6-5 Explain how the hematocrit level is calculated on automated hematology instruments.

- 6-6 Analyze the significance of red blood cell indices as related to the diagnosis of anemia.
- 6-7 Describe the appearance of the peripheral blood smear in various anemias.
- 6-8 Explain the diagnostic value of the reticulocyte count.
- 6-9 List factors to be evaluated in the interpretation of a bone marrow aspirate smear.
- 6-10 Explain the significance of the new RBC parameters in testing for anemia.
- 6-11 Describe the general approach to treatment of anemia.

Anemia is a condition in which there is a decrease in the oxygen-carrying capacity of blood. This results in a deficiency and an inability of the circulating blood to supply the tissue with adequate oxygen for proper metabolic function.¹ Anemia is usually associated with decreased levels of red blood cells (RBCs), hemoglobin (Hgb), and packed RBC (PRBC), more commonly known as hematocrit (Hct).¹ Clinically, the diagnosis of anemia is made by patient history, physical examination, signs and symptoms, and hematologic laboratory findings. It is the most common hematologic disorder nationally and internationally.²

There are varied types and causes of anemias. Anemia is often the symptom of a more serious underlying disease. Determining the specific cause of an anemia in an individual is important in determining the appropriate therapy and assessing the prognosis of the disease in that patient. From a practical laboratory standpoint, the typical diagnostic criterion for anemia is a decreased hemoglobin (Hgb), hematocrit, or RBC count.³

Because most patients with anemia have lowered hemoglobin levels, anemia is defined as a Hgb <12 g/dL in females and <14 g/dL in males. Anemia may be further classified arbitrarily as either moderate (7 to 10 g Hgb/dL) or severe (<7 g Hgb/dL).³ Moderate anemias typically do not produce clinically evident signs or symptoms, especially if the onset is slow. However, depending on the patient's age or cardiovascular condition, even a moderate degree of anemia may be associated with exertional dyspnea (difficulty breathing), light-headedness, vertigo, muscle weakness, headache, or general lethargy. Anemia of rapid onset, such as that resulting from gastrointestinal hemorrhage, may be associated with significant clinical symptoms, such as hypotension, tachycardia, and dyspnea. These symptoms are usually associated with the precipitous loss of intravascular volume as well as the oxygen-carrying capacity of the RBCs. Another example of acute massive blood loss is hemorrhage from extensive

trauma or major surgery. With normal compensatory mechanisms, the rate of RBC production equals the rate of RBC loss. When the rate of RBC production decreases or the rate of RBC loss increases, anemia develops.

Causes, Considerations, and Compensatory Mechanisms

Anemia has many causes; Box 6-1 broadly classifies the categories as blood loss, accelerated destruction of RBCs, nutritional deficiency, bone marrow replacement, infection, toxicity, hematopoietic stem cell arrest or damage, and hereditary or acquired defects of hemoglobin, RBC enzymes, or other metabolic abnormalities. Categories may be simplified to include conditions of increased RBC destruction, abnormal or decreased RBC production, or some combination thereof.

Normal levels of red blood cells and their associated parameters (e.g., hemoglobin and hematocrit) are dependent on a person's age and sex, as well as other factors.

The reference range for hemoglobin in newborn infants (younger than 1 week old) is 18 ± 4 g/dL. Adult hemoglobin reference ranges are approximately 16.0 ± 2 g/dL for men and 14.0 ± 2 g/dL for women (Table 6-1). In the geriatric age group, the difference between hemoglobin levels of men and women narrows. The hemoglobin levels of geriatric men usually decrease slightly, and those of postmenopausal women approach those of men. The reference ranges used by each laboratory should be followed, because these values depend on the institution and the patient population served.

Various diseases and disorders are associated with lower than usual hemoglobin levels; these include nutritional deficiencies,⁴ external or internal blood loss, accelerated destruction of RBCs, ineffective or decreased production of RBCs,⁵ abnormal hemoglobin synthesis, bone marrow replacement by infection or tumor, and bone marrow suppression by toxins, chemicals, or radiation.³

CRITICAL THINKING QUESTION

6-1 What two factors affect the reference ranges for each laboratory?

See answers to all Critical Thinking Questions at the back of this book.

BOX 6-1 Causes of Anemia by Category

- Blood loss (hemorrhage, surgery, menses)
- Accelerated destruction of RBCs (immune and nonimmune hemolytic)
- Nutritional deficiency (folate, vitamin B₁₂, iron)
- Bone marrow replacement (cancer, infection)
- Infection (viral, bacterial, microbacterial, mycobacterial, parasitic)
- Toxicity (hydrocarbons, medications, radiation)
- Hematopoietic stem cell arrest or damage (aging, cytokines)
- Hereditary or acquired defect (glucose-6-phosphate dehydrogenase [G6PD] deficiency, spherocytosis)
- Unknown/idiopathic

TABLE 6-1 Reference Range Values for Hemoglobin by Age Group

Age Group	Hemoglobin (g/dL)
Infants and Children	
Newborns (<1 week old)	14.0–22.0
6 mo old	11.0–14.0
Children (1–15 years old)	11.0–15.0
Adults	
Men	14.0–18.0
	WHO 15.2 ± 0.9
Women	12.0–16.0
	WHO 13.3 ± 0.9

WHO = World Health Organization

Note this table data is based on studies from eight countries for WHO values. Reference ranges should be established for each laboratory based on the population served.

In a healthy ambulatory person, approximately 1% of the senescent circulating RBCs are lost daily. Normally, the bone marrow continues to produce RBCs. A laboratory measure of this replacement is the reticulocyte count.⁶ Bone marrow production of new RBCs in response to proper nutrients, vitamins, and other factors can be evaluated by the reticulocyte count.

In healthy adults, reticulocytes, which are early circulating RBCs containing residual RNA, account for 0.5% to 2.0% of the circulating RBCs. Replacement of RBCs requires a bone marrow with adequate functioning stem cells. In addition, normal RBC maturation, development, and the ability to release mature RBCs from the bone marrow are essential in this process. Proper hemoglobin and RBC production require a variety of nutritional factors, including iron, vitamin B₁₂, folic acid, and normal pathways of hemoglobin synthesis. Decreased production of hemoglobin and/or RBCs results in a hypoproliferative anemia. The role of hemoglobin synthesis in anemias is covered in greater detail in Chapter 7: Iron Metabolism and Hypochromic Anemias.

ADVANCED CONTENT

In severe anemias (less than 7 g Hgb/dL), symptoms of functional impairment of several organ systems may be evident. For example, with minimal exercise, a patient's cardiac and respiratory rates may increase dramatically.⁷ If the anemia is secondary to blood loss and decreased intravascular volume, a patient's blood pressure may drop significantly when rising from the reclining to a sitting or standing position. Heart rate will increase in an effort to elevate cardiac output to keep pace with peripheral tissue oxygen demands in the face of a decreased oxygen-carrying capacity of the lowered hemoglobin level. Respiratory symptoms, including dyspnea on exertion, may also occur with anemia.⁷

An interesting compensatory mechanism in response to anemia is an increase in the 2,3-diphosphoglycerate (2,3-BPG) level.⁸ This compound is a remarkable physiological regulator of normal hemoglobin oxygen-carrying capacity and tissue oxygen delivery. In the presence of 2,3-BPG, hemoglobin can more readily release the oxygen it is carrying to peripheral tissues. This enhanced release occurs regardless of blood arterial oxygen level.

A healthy individual responds to anemia with elevated levels of erythropoietin (Epo), a hormone with molecular mass of approximately 31,000 daltons.⁹ The Epo level is sometimes used as an ancillary diagnostic aid in the differential diagnosis of anemia. It has a plasma half-life of between 6 and 9 hours and is produced by the peritubular complex of the kidney. Epo levels vary as a result of altered oxygen tension in the tissues of the kidney. Increased Epo production occurs when there is a decreased hemoglobin level, a hemoglobin structural problem in which oxygen is not released, or low ambient oxygen tension at high altitude. On the other hand, high oxygen levels in the kidney result in a decrease in Epo production.

Other factors, including geographic elevation, influence individual "normal" hemoglobin levels. Persons living at elevations above 8,000 feet may have persistently increased hemoglobin values secondary to decreased oxygen saturation in the ambient atmosphere. Lung diseases may alter oxygen diffusion across the lung alveolar membranes. A compensatory sequel to chronic lung disease may result in increased hemoglobin levels resulting in secondary polycythemia⁷ (see Chapter 19: Chronic Myeloproliferative Disorders: Polycythemia Vera, Essential Thrombocythemia, and Idiopathic Myelofibrosis).

Clinical Diagnosis of Anemia

The clinical diagnosis of anemia is made using a combination of factors, including patient history, physical examination to identify signs and symptoms, and changes in the patient's hematologic profile. The signs and symptoms of anemia are generally nonspecific, such as fatigue and weakness, and may include gastrointestinal symptoms, such as nausea, constipation, and diarrhea.⁸ The patient may complain of **dyspnea** (difficulty breathing) after a level of exertion that previously had not caused any problems. The common clinical symptoms of anemia are listed in Box 6-2.

A patient example may be useful here: A man who had been able to climb three flights of stairs without difficulty or significant shortness of breath reports that now he must stop after climbing one flight of stairs because he is very short of breath. Subsequent information indicates that the patient has passed **melena** (very dark stools) over the past week. Measurement of his hemoglobin reveals a level of 8 g/dL. The initial diagnostic impression from the clinical information is that the patient's anemia is secondary to gastrointestinal bleeding.

Physical signs of anemia typically are not specific to the underlying causative disease. Occasionally, however, the

BOX 6-2 Clinical Symptoms of Anemia

- Pallor
- Weakness
- Fatigue
- Lethargy/malaise
- Exercise dyspnea
- Palpitation
- Pica (consumption of substance such as ice, starch, or clay; frequently found in iron-deficiency anemia)
- Syncope (particularly following exercise)
- Dizziness/vertigo
- Headache
- Tinnitus
- Irritability
- Difficulty sleeping
- Difficulty concentrating
- Gastrointestinal symptoms

etiology may be suspected from certain physical findings. For example, severe pallor, smooth tongue, and an esophageal web in a patient with severe iron-deficiency anemia⁹⁻¹¹ could point to a loss of blood due to GI bleeding (see Chapter 7: Iron Metabolism and Hypochromic Anemias). Light-skinned patients who are anemic may appear to have pale coloration of mucosal membranes, nailbeds, and skin. Occasionally, patients with anemia have slightly elevated temperatures, particularly in certain types of hemolytic anemia. In the presence of anemia, heart murmurs may be heard; these can be secondary to the cause of the anemia and related to the increased cardiac workload required to bring oxygen to the tissues.⁸

A broad category of anemia includes those termed *aplastic*. **Aplastic anemia** is defined as **pancytopenia** involving red blood cells, white blood cells, and platelets, and resulting from bone marrow production failure.¹² **Hypoproliferative** anemia can be solely RBC-associated. Additional information concerning aplastic anemia is found in Chapter 13: Rare Normocytic Normochromic Anemias: Aplastic, Pure Red Cell Aplasia, Congenital Dyserythropoietic Anemia, and Paroxysmal Nocturnal Hemoglobinuria.

ADVANCED CONTENT

In general, there are two types of aplastic anemia. The first type includes congenital or so-called idiopathic forms.¹² The second type is secondary or acquired.¹² Causes of secondary aplastic anemia include exposure to chemicals (e.g., benzene and some other fluorocarbons), therapeutic agents (especially cancer chemotherapy agents), infection (e.g., some types of hepatitis and parvovirus infections), and ionizing radiation.¹² Aplastic anemia is a pancytopenia involving RBCs, white cells, and platelets.

Anemia can also be found in patients with bacterial endocarditis who also have fever and heart murmurs. Bacterial endocarditis is a clinical example in which the damaged,

infected myocardial valve and heart murmur are related etiologically to the anemia.⁷ Prosthetic heart valves, arterial grafts, and disseminated intravascular coagulation (DIC) can cause a form of mechanical hemolytic anemia known as **microangiopathic hemolytic anemia**.⁷ This anemia is a subgroup of hemolytic anemia resulting in fragmentation and destruction of RBCs caused by diseases of small blood vessels.

CRITICAL THINKING QUESTION

6-2 What components in the blood are affected in aplastic anemia?

Classification of Anemia

The individual types of anemias can be classified according to several different criteria. A bone marrow dynamic classification is **hypoproliferative** (decreased production), **hemolytic** (accelerated destruction), or a combination of the two (sometimes called **ineffective hematopoiesis**). Anemias are often classified clinically according to their associated causes, such as blood loss, iron deficiency, hemolysis, infection, metastatic bone marrow replacement, and nutritional deficiency (see Box 6-1).

Laboratory Classification of Anemias

Anemias can also be categorized quantitatively in the laboratory by their hematocrit and hemoglobin levels, blood cell indices, and/or reticulocyte count.¹³⁻¹⁵ Laboratory scientists are frequently involved in the quantitative measurements that lead to these classifications and in subsequent evaluations (see Figure 6-1).

Hemoglobin and Hematocrit Levels

Measurement of the hemoglobin and hematocrit levels is the usual method of determining whether a patient has anemia. Hemoglobin is the main component of the RBC. It is the physiological carrier of oxygen to tissues and acts as a buffer to handle carbon dioxide formed in metabolic activities.⁸ The hematocrit, or packed RBC volume, is the ratio of the volume of RBCs to the volume of whole blood. Hematocrit is usually expressed as a percentage (e.g., 42%), but is expressed in Standard International (SI) units as a decimal fraction (e.g., 0.42 L/L).

Several companies manufacture automated instruments that include the determinations of hemoglobin and hematocrit levels as part of a hematologic profile known as the complete blood count (CBC). The Hct reference range for adult men is $47\% \pm 5\%$, although it does vary by institution and instrumentation. For adult women during the reproductive years, the Hct reference range is $42\% \pm 5\%$. Different reference ranges are required for neonates and very young children, and, as with all reference ranges, vary among institutions and patient populations.⁸

A general rule or check on the reported Hgb and Hct levels is that the Hct should be approximately three times the Hgb

value, or the ratio of Hgb to Hct is 1:3. This ratio may vary with the cause of the anemia and the effect of that cause on the RBC indices.

Problems in the measurement of hematocrit include a high RBC count (polycythemia) and/or a high reticulocyte or WBC count, because hematocrit determinations using hematology analyzers can result in the calculation of falsely elevated values. The higher cell volumes of these cells and increased numbers can interfere with the mean corpuscular volume (MCV) and red blood cell count during the calculation of the hematocrit. Figure 6-1 is a simplified algorithm for anemias using Hgb, Hct, and RBC indices.

Histologic examinations of the bone marrow smear and aspirate are adjuncts to further elucidate the cause of the anemia.

Morphological Classification of Anemias

Classifying a disorder by the size and appearance of RBCs is known as morphological classification. To classify anemias, red blood cell indices are measured and/or calculated.

Red Blood Cell Indices

RBC indices include the mean corpuscular volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC).¹⁰ The MCV is the average volume, expressed in femtoliters (fL), of RBCs. It is measured directly or calculated from the hematocrit and RBC counts. The MCH is the content of hemoglobin in the average RBC. MCH is calculated from the hemoglobin concentration and the RBC count. MCHC, the average concentration of hemoglobin in a volume of packed RBCs, is calculated from the hemoglobin concentration and the hematocrit level.¹⁰

RBC indices are readily available from automated hematology counting devices. Values are calculated in devices in which the MCV is derived from the voltage changes formed during the RBC count. The calculations are:

- Hematocrit equals the MCV times the RBC count
- MCH equals the hemoglobin divided by the RBC count
- MCHC equals the hemoglobin divided by the hematocrit

The adult reference range for MCV is 80 to 100 fL; for MCH it is 27 to 31 pg; and for MCHC it is 32.0 to 35.9 g/dL.

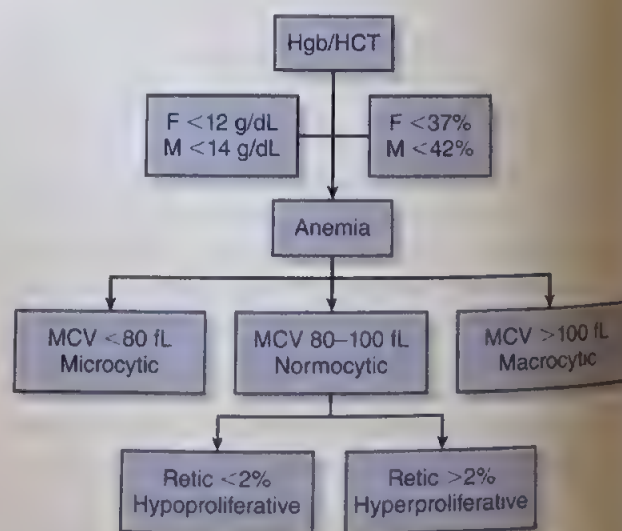


FIGURE 6-1 Simplified algorithm for anemias using RBC indices.

(32% to 36%). If the MCV is within the reference range, the RBCs are considered **normocytic**; if less than normal, they are **microcytic**; and if greater than normal, they are **macrocytic**. Both MCH and MCHC values are used to determine the content of hemoglobin in RBCs. The MCH strongly correlates with the MCV.¹³

In various anemic states, the indices may be altered as follows: In microcytic anemia, from an MCV of less than 80 fL down to a low of approximately 50 fL; from an MCH of less than 25 pg to approximately 15 pg; and from an MCHC of less than 30% to 22%. In the macrocytic anemias, MCV values are usually greater than 100 fL and may be as high as, and sometimes even higher than, 120 fL, and the MCHC may be normal or decreased. The MCHC may be increased only in the presence of spherocytosis, if at all.

Most automated hematology instruments also provide an RBC distribution width (RDW) value. The RDW is an index of RBC size variation that can be used to quantitate the amount of anisocytosis seen on a peripheral blood smear. The reference range for RDW is 11.5% to 14.5% for both men and women.

Calculation of Red Blood Cell Indices

The RBC indices are accurately calculated by automated hematology instrumentation. These instruments provide precise numeric values for hemoglobin levels, the numbers of RBCs, and the MCV. Although less precise, careful microscopic examination of a peripheral blood smear can determine whether the RBCs are normocytic, microcytic, macrocytic, normochromic (normal amount of hemoglobin), or hypochromic (lower amount of hemoglobin). A proper specimen is required to obtain accurate answers.¹¹

RBC indices equations and reference ranges are shown in Box 6-3.

Use of the RBC indices in the differential diagnosis of anemia can provide a general idea as to what is occurring clinically (Table 6-2). A normocytic normochromic anemia may be the result of bone marrow failure, hemolytic anemia, or some subset of either of these conditions.⁷

BOX 6-3 RBC Index Equations and Reference Ranges

MCV equals Hct (in %) multiplied by 10, divided by RBC count (in millions/ μ L); reference range: 90 ± 10 fL.

$$\text{MCV} = \frac{\text{Hct (\%)} \times 10}{\text{RBC count (millions}/\mu\text{L})}$$

MCH equals Hgb (in g/dL) multiplied by 10, divided by RBC count (in millions/ μ L); reference range: 29 ± 2 pg.

$$\text{MCH} = \frac{\text{Hgb (g/dL)} \times 10}{\text{RBC count (millions}/\mu\text{L})}$$

MCHC equals Hgb (in g/dL) multiplied by 100, divided by Hct (as a percentage); reference range: $34 \pm 2\%$.

$$\text{MCHC} = \frac{\text{Hgb (g/dL)} \times 100}{\text{Hct (\%)}}$$

TABLE 6-2 Morphological Classification of Anemias by RBC Indices

Size (MCV) (fL)	Hgb Content (MCHC) (%)	Possible Causes
Normocytic (80–100)	Normochromic (32–36)	<ul style="list-style-type: none"> • Bone marrow failure • Hemolytic anemia • Chronic renal disease • Leukemia • Metastatic malignancy
Macrocytic (>100)	Normochromic (32–36)	<ul style="list-style-type: none"> • Megaloblastic and nonmegaloblastic macrocytic anemias (e.g., liver disease, myelodysplasias)
Microcytic (<80)	Hypochromic (<32)	<ul style="list-style-type: none"> • Iron deficiency • Sideroblastic anemia • Thalassemia • Lead poisoning • Chronic diseases • Chronic infection or inflammation • Unstable hemoglobins

Other Laboratory Tests

Other laboratory tests used to classify anemias include reticulocyte count, peripheral blood smear, and bone marrow smear and biopsy.

Reticulocyte Count

The differential diagnosis of bone marrow failure requires information about RBC production. This information can be obtained from the reticulocyte count, which indicates whether there is bone marrow capacity for increased RBC production. Because RBC destruction may exceed production, the reticulocyte count, in fact, measures effective RBC production.

Reticulocytes are nonnucleated RBCs that still contain RNA. Reticulocytes can be visualized after incubation with a variety of so-called supravital dyes, including crystal violet and brilliant cresyl blue.

In Figure 6-2, reticulocytes can be visualized using a new methylene blue stain.

RNA is precipitated as a dye-protein complex. Under normal circumstances, reticulocytes lose their RNA a day or so after reaching the bloodstream from the marrow. Reticulocyte activity can be expressed as an absolute count, a production index, or a percentage.¹⁴ Because anemia typically is accompanied by increased bone marrow activity, the reticulocytes are expected to increase. A corrected reticulocyte count compares the patient hematocrit value with a normal Hct value using the following formula:

$$\text{Corrected reticulocyte \%} = \frac{\text{Patient Hct \%} \times \text{Reticulocyte \%}}{\text{Reference Hct mean (45 \%)}}$$

For example, in a patient presenting with a reticulocyte count of 10% with a hematocrit of 22%, the correct reticulocyte would be:

$$\frac{10\% \times 22\%}{45\%} = 4.9\%$$

The reticulocyte count can also be expressed as the reticulocyte production index (RPI). The RPI accounts for the presence of “stress or shift” reticulocytes in the peripheral blood. These premature reticulocytes contain more filamentous reticulum and result in a longer maturation time in circulation. The RPI calculation takes into account the corrected reticulocyte count as well as the maturation time of the reticulocyte based upon the patient hematocrit.

The RPI is calculated as follows:

$$\frac{[\text{Reticulocyte count (\%)} \times \text{Patient Hct (\%)}] / 45(\%)}{2 \text{ (Stress reticulocyte maturation time at 25\% Hct)}}$$

For example, a patient with a reticulocyte count of 12% and a hematocrit of 25% would yield an RPI of:

$$\frac{(12\% \times 25\%) / 45\%}{2} = 3.3$$

Interpretation of the reticulocyte count must take into account the age and nutritional status of the patient. The reference range for healthy adults is a reticulocyte count between 0.5% and 2.0%, or from 24 to 100 × 10⁹ reticulocytes per liter. Newborn infants have a higher reticulocyte count of 2.5% to 6.0%.

A high reticulocyte count is characteristic of hemolytic anemia when there is decreased RBC survival. This may be the result of extravascular elimination, intravascular destruction, or a combination of the two.¹⁵ Macrocytic normochromic anemias usually occur in association with folate or vitamin B₁₂ deficiency.¹⁶ The most commonly encountered anemias are the microcytic hypochromic anemias, usually referred to as iron-deficiency anemia (IDA).^{10,17} In IDA, the reticulocyte is decreased in relation to the severity of the anemia as a result of ineffective erythropoiesis. Beta-thalassemia, an inherited defect of hemoglobin synthesis, is another cause of microcytosis and decreased reticulocyte count.⁵ Less frequently seen are the sideroblastic anemias, which are also associated with decreased MCV and reticulocyte count.¹⁰ These anemias are described in more detail in Chapters 7 through 15.

CRITICAL THINKING QUESTION

6-3 In what type(s) of anemia would the reticulocyte count be decreased?

ADVANCED CONTENT

Other Factors Involved in Red Blood Cell Production

Bone marrow stem cells require various other factors to continue to synthesize hemoglobin and permit proper maturation

of erythrocytes and other cells. These include cytokines: for example, stem cell factor, erythropoietin, interleukin-3 (IL-3), and other interleukins. The hormones of the androgen group and thyroxine are also necessary. Many vitamins such as vitamin B₁₂, folate, vitamin C, vitamin E, pyridoxine (B₆), thiamin, riboflavin, and pantothenic acid, play roles in bone marrow productivity.⁸ Certain metals, such as iron, manganese, and cobalt, are part of the production process for RBCs and other bone marrow cellular components.⁸

Some factors act as growth-inhibiting agents. Among these is transforming growth factor-beta (TGF-β), which causes inhibition of growth of hematopoietic stem cells. Myeloid progenitors are known to be inhibited by tumor necrosis factor (TNF) and interleukin-4 (IL-4).⁸

CRITICAL THINKING QUESTION

6-4 What laboratory tests are used to diagnose anemia?

Peripheral Blood Smear

Microscopic examination of a properly collected and prepared peripheral blood smear is a requirement for the clinical and laboratory evaluation of anemia. A peripheral blood smear can provide much information concerning the cause of an anemia. For example, coexistent neutropenia, thrombocytopenia, and anemia may indicate bone marrow failure or a lack of a nutritional substance to allow for adequate bone marrow production.¹⁵ The size and shape of the RBCs can be noted by the examiner as part of the peripheral blood smear. Variations in the size of RBCs result in **anisocytosis**, and variations in their shape result in **poikilocytosis**. The hemoglobin (chromatin) content of the RBCs can be inspected visually on the peripheral smear. In addition, morphological cellular details on the peripheral smear may provide clues to the etiology of the anemia, the bone marrow response, or both. The white cells may be evaluated; for example, excess lobulations of the polymorphonuclear leukocytes are seen in the hypersegmented granulocytes of macrocytic anemias (see Chapter 8: Megaloblastic Anemias and Other Macrocytic Anemias).

Basophilic stippling in the RBCs may suggest the presence of increased bone marrow production and reticulocytosis (Fig. 6-2). It may also indicate that there are remnants of RNA, which may be associated with lead poisoning and some malignancies. Howell-Jolly bodies are small, round, blue inclusions seen in RBCs as the result of fragments of DNA still remaining in the cell (see Chapter 4: Examination of the Peripheral Smear: Red Cell, White Cell, and Platelet Morphology). Howell-Jolly bodies are often seen in hyposplenism and asplenism, pernicious anemia, and some hemoglobinopathies—particularly thalassemia.^{8,9} Pappenheimer bodies, which are iron-containing or siderotic granules, appear as purplish-blue granules with Wright's stain and as coarse blue granules with Prussian blue iron stain. The clinical disorders associated with Pappenheimer bodies include sideroblastic anemia, alcoholism, thalassemia, and some myelodysplastic syndromes.^{8,17} Nucleated RBCs with



FIGURE 6-2 Reticulocytosis (new methylene blue stain).

iron granules are known as sideroblasts, and RBCs containing iron granules but without a nucleus are referred to as siderocytes. Ringed sideroblasts are those in which more than five granules are arranged in a ring around the nucleus of an orthochromatic normoblast (Fig. 6-3). Ringed sideroblasts are indicative of ineffective erythropoiesis.

A threadlike blue ring contained entirely within an abnormal RBC, which may or may not have a “figure-of-8” and a round or oval configuration, is known as a Cabot ring (see Chapter 4), which is a remnant of the nuclear membrane. This infrequent finding may be seen in several clinical disorders, including pernicious anemia, lead poisoning, and other severe anemias.⁸ Heinz bodies (Fig. 6-4) are small, rounded, angular inclusions about one micron in diameter and represent aggregates of denatured hemoglobin that are negative when stained with Prussian blue or other iron stains. Heinz bodies can be demonstrated only by using supravital stains (e.g., crystal violet) and are not visible with the usual Wright’s stain. The clinical disorders that have been associated with Heinz bodies include glucose-6-phosphate dehydrogenase (G6PD) deficiency after exposure to oxidizing drugs, unstable hemoglobinopathies, alpha-thalassemia, and post-splenectomy.⁸

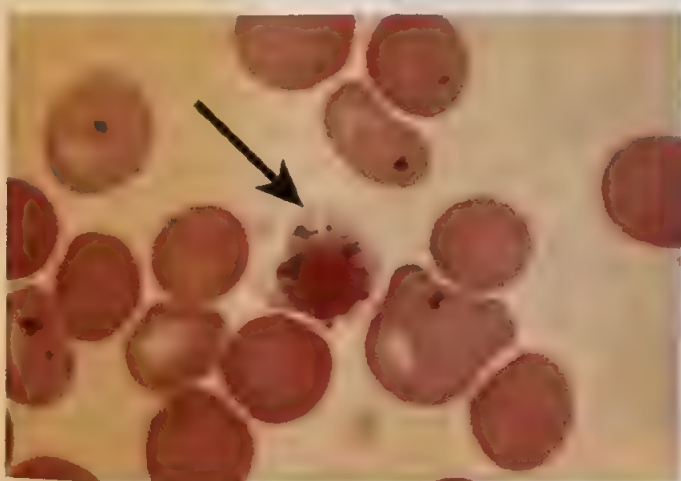


FIGURE 6-3 Ringed sideroblast (center) and siderocytes (surrounding cells). (From Bell A. Hematology, Listen, Look and Learn. Bethesda, MD: Health and Education Resources, Inc., with permission.)



FIGURE 6-4 Heinz bodies. (From Bell A. Hematology, Listen, Look and Learn. Bethesda, MD: Health and Education Resources, Inc., with permission.)

Bone Marrow Smear and Biopsy

Bone marrow aspiration and biopsy are important diagnostic tools in the determination of anemia. Bone marrow interpretation and evaluation are covered in Chapter 3: Bone Marrow Structure and Function. Factors to be evaluated in interpretation of a bone marrow aspirate smear and biopsy include maturation of the red and white cell series, presence of megakaryocytes, ratio of myeloid to erythroid cells, abundance of iron stores, presence or absence of granulomas, tumor cells, and overall estimate of bone marrow cellularity.

Interpretation of the bone marrow requires a differential count of the myeloid, lymphoid, and erythroid series; an iron stain; and other appropriate techniques. If a differential diagnosis of lymphoproliferative or myeloproliferative disorders is being considered, immunohistochemical stains typically are performed. Other appropriate specific stains may be indicated if metastatic tumor or infection is suspected or being evaluated.¹⁸

Differential Diagnosis

The differential diagnosis of anemia is based on a combination of laboratory findings (see Table 6-2) and clinical symptoms (see Box 6-2). A decision-making flowchart for the diagnosis of anemia using RBC indices is provided in Figure 6-5.

A wide variety of marrow, extramedullary, and interrelating disease states requires careful consideration in the differential diagnosis of anemia. Many tests are available to aid in the differential diagnosis of the multiple types of anemia. Successful study of the causes of anemia requires a broad knowledge of clinical laboratory testing. The other tests that may be performed in the diagnosis of anemias and the chapters in which these tests are discussed are listed in Table 6-3.

New RBC Parameters in Testing for Anemia

The latest generation of hematology analyzers provides new RBC parameters and reticulocyte indices.^{6,13,14} These include the percentage of microcytic RBCs, the percentage of macrocytic RBCs, the percentage of hypochromic

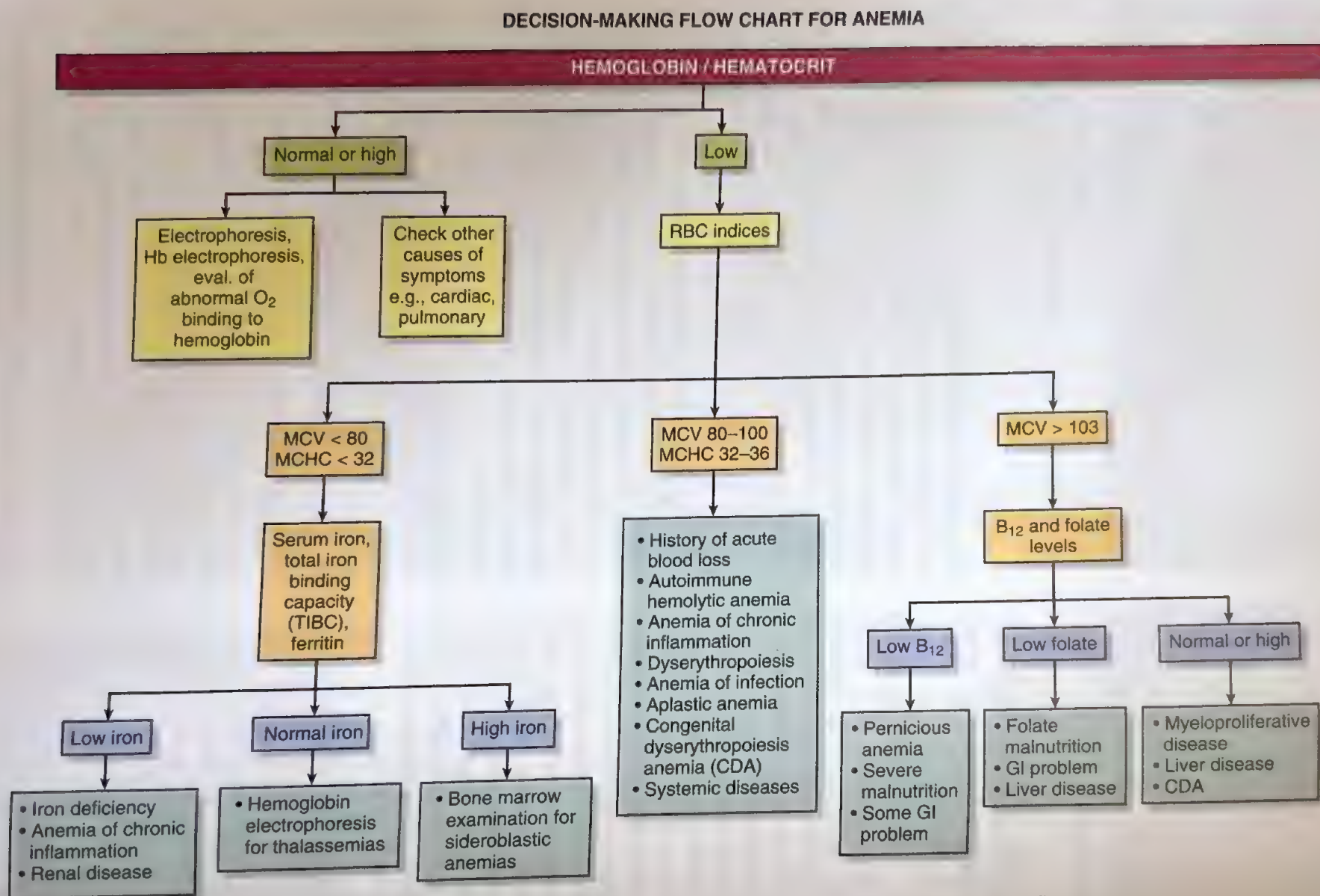


FIGURE 6-5 Decision-making flowchart (algorithm) for the diagnosis of anemia using the RBC indices.

TABLE 6-3 Other Tests That May Be Performed in the Diagnosis of Anemias

Test	Diagnostic Use
Hemoglobin electrophoresis	<ul style="list-style-type: none"> Hemoglobinopathies Thalassemia syndromes
Antiglobulin testing	<ul style="list-style-type: none"> Hemolytic anemias
Osmotic fragility test	<ul style="list-style-type: none"> Hereditary spherocytosis Severe iron deficiency Sickle cell disease β-Thalassemia
Tests for red blood cell enzymes	<ul style="list-style-type: none"> Hemolytic anemias glucose-6-phosphate dehydrogenase (G6PD) deficiency pyruvate kinase (PK) deficiency
Serum iron and iron-binding capacity	<ul style="list-style-type: none"> Iron-deficiency anemia
Folate and vitamin B ₁₂ measurements	<ul style="list-style-type: none"> Megaloblastic anemias

*Primary use

RBCs, the percentage of hyperchromic RBCs, the reticulocyte hemoglobin content, and the immature reticulocyte fraction.⁶ Several studies have demonstrated the usefulness of these new parameters in selected patient populations.^{6,13,18,20} It has been reported that, when these new parameters are combined, they provide information at a cellular level regarding iron availability in RBCs and reticulocytes.⁶ The most widely studied parameter, the hemoglobin content of the reticulocyte, allows for early detection of iron-deficiency anemia.⁶ The major limitation of these new parameters is the difficulty in comparing results from the different manufacturers of automated analyzers.¹³ Another variable or limitation is the storage time and temperature of the samples used to determine these new RBC parameters.⁶ Table 6-4 lists the different designations for each of these new RBC parameters by the type of automated analyzer, and Table 6-5 outlines the new red blood cell parameters.

ADVANCED CONCEPTS

The percentage of mature hypochromic RBCs reflects the iron status of the patient's red cells during circulation.

TABLE 6-4 Designations for New RBC Parameters by Analyzer

New RBC Parameter	Sysmex Xn 9000	Abbott Sapphire	Siemens Advia 2120
Percentage of microcytic RBCs	% Micro-R	% MIC	Micro %
Percentage of macrocytic RBCs	% Macro-R	% MAC	Macro %
Percentage of hypochromic RBCs	% Hypo-He	% HPO	Hypo %
Percentage of hyperchromic RBCs	% Hyper-He	% HPR	Hper%
Reticulocyte hemoglobin content	Retic-He	MCHr	CHr
Immature reticulocyte fraction	IRF	IRF	IRF

Based on the new RBC parameters, the ratio index of percent microcytic to percent hyperchromic has been shown to be more sensitive as a screening tool for distinguishing between iron deficiency and thalassemia, compared with the traditional indices.⁶

In addition, the percent microcytic RBCs is greater in thalassemia compared with iron-deficiency anemia.⁶

The percent hypochromic red blood cells has also been useful in the anemia of chronic kidney disease by identifying iron-restricted erythropoiesis in anemic patients treated with erythropoiesis stimulating agents.¹³ The percent hyperchromic cells is useful in the diagnosis of hereditary spherocytosis and immune hemolytic anemia.¹³

It has been reported that determination of the hemoglobin content of reticulocytes has several advantages for routine use.⁶ Unlike traditional chemistry tests, this parameter is not influenced by infection or inflammation and can be performed on the same sample used for the complete blood count.⁶

New red cell parameters and reticulocyte indices will play an important role in the differential diagnosis and monitoring of anemias. However, it should be remembered that microscopic examination of the peripheral smear is essential, despite the advancement of newer generations of automated hematology analyzers.

TABLE 6-5 New Red Blood Cell Parameters

Variable	Clinical Application	Limitations
Percentage of hypochromic red cells	Assessment of iron availability (absolute or functional) for erythropoiesis. Related to iron status in the last 3 months.	Affected by preanalytical variables (storage temperature, time). Reference intervals and diagnostic thresholds are method dependent. Limited value in the presence of β thalassemia.
Percentage of hyperchromic red cells	Diagnosis of hereditary/immune spherocytosis	Reference intervals and diagnostic thresholds are method dependent.
Percentage of microcytic red cells	Useful in combination with other RBC parameters (mainly hypochromic erythrocytes) to obtain discriminant indices for the differential diagnosis of microcytic anemia.	Reference intervals and diagnostic thresholds are method dependent.
Reticulocyte Parameters		
Immature reticulocyte fraction (IRF) (fraction)	Classification of anemias and monitoring of treatment. Verify aplastic anemia.	Reference intervals and diagnostic cutoff are method dependent.
Reticulocyte mean hemoglobin content (pg)	Diagnosis of iron-deficient erythropoiesis. Early monitoring the response to iron therapies.	Limited value in the presence of α - or β -thalassemia.
Mean reticulocyte volume (fL)	Diagnosis of iron-deficient erythropoiesis. Early monitoring of treatment with B_{12} /folate/iron in nutritional anemia.	Affected by preanalytical variables (storage temperature, time). Reference intervals strictly method dependent.

Modified from Buttarello M. Laboratory diagnosis of anemia: are the old and new red cell parameters useful in classification and treatment, how? *Int J Lab Hematol*. 2016 May 38(Suppl 1):123-32.

Overview of the Treatment of Anemia

Because anemia is treated according to its cause(s), the cause should be considered and determined before beginning either supportive therapy (such as a transfusion) or replacement therapy. This chapter has identified only some of the possible causes of anemia. Indeed, more than one cause of anemia can exist in a patient. Obtaining the proper diagnostic studies in the shortest and most cost-effective manner is the responsibility of the attending physician and laboratory professionals.

ADVANCED CONTENT

The natural history of anemia depends on its cause. For example, a patient with an iron-deficiency anemia associated with carcinoma of the colon may present with signs of an iron-deficiency anemia associated with blood loss from the tumor.⁷ Later, with more extensive tumor involvement, the anemia may include a bone marrow failure component because of bone marrow replacement by the tumor, as in a **myelophthisic** anemia. Patients with pernicious anemia require a lifetime of parenteral vitamin B_{12} supplementation.¹⁷

whereas those with other forms of megaloblastic anemia may require only a balanced diet and replacement of folic acid.¹⁷

Transfusions can obscure and confuse the findings of diagnostic tests in patients with anemia. Transfusions can suppress erythropoiesis; alter vitamin B_{12} , folate, and iron levels; and thwart the interpretation of diagnostic tests seeking the specific cause of the anemia.¹⁵ It is important that a diagnosis be made, if at all possible, before transfusions are given.

Recombinant Epo is now available for treatment of certain types of anemias, particularly end-stage renal disease anemia associated with human immunodeficiency virus and anemia associated with cancer and certain other chronic disorders.⁷

CRITICAL THINKING QUESTION

6-5 Why is it important to determine the cause of anemia before administering treatments such as transfusion?

SUMMARY CHART

- There are many causes of anemia, including nutritional deficiencies, blood loss, increased destruction or decreased production of RBCs, infections, toxicity, heredity, and acquired defects.
- Anemia is usually characterized by decreased RBC count, hemoglobin, and/or hematocrit levels.
- Hemoglobin levels vary with age and sex. Reference ranges should be determined and reported for infants, children, and adult men and women.
- Clinical symptoms associated with anemia may include vertigo, light-headedness, muscle weakness, headache, or dyspnea.
- Adult hemoglobin reference ranges are approximately 14 to 18 g/dL for men and 12 to 16 g/dL for women.
- The hematocrit (Hct), or packed red cell volume, is determined by centrifugation of blood of either capillary or venous origin; it can be calculated by the mean corpuscular volume (MCV) multiplied by the RBC count and divided by the sample size.
- The hematocrit reference range of adult males is $47\% \pm 5\%$; for women, it is $42\% \pm 5\%$.
- RBC indices include the mean corpuscular volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC); their reference range values can be used to aid in the morphological classification and diagnosis of anemias.
- Morphological examinations of the peripheral blood smear and bone marrow preparations are valuable aids in the diagnosis of anemias.
- Anemias may be classified as normocytic and normochromic, microcytic and hypochromic, or macrocytic and normochromic.
- The reticulocyte count is useful in determining the response and potential of the bone marrow. Normal adult values are 0.5% to 2.0%. The count can be determined by automated or manual methods.
- The corrected reticulocyte count is determined by patient packed corpuscular volume (Hct) multiplied by the reticulocyte count (%), divided by the reference Hct.
- Bone marrow aspiration and biopsy are used in the overall estimate of bone marrow cellularity.
- The latest generation of hematology analyzers provides new RBC parameters and reticulocyte indices, including the percentages of microcytic RBCs, macrocytic RBCs, hypochromic RBCs, and hyperchromic RBCs, as well as the reticulocyte hemoglobin content, and the immature reticulocyte fraction.
- Because anemia is treated according to its cause(s), the cause should be considered and determined before beginning either supportive therapy (such as a transfusion) or replacement therapy.

REVIEW QUESTIONS

1. Which hematology test can you use to monitor the response of the bone marrow?
 - a. MCV
 - b. RDW
 - c. Platelet count
 - d. Reticulocyte count
2. Which of the following conditions can be a cause of anemia?
 - a. Dehydration
 - b. Moderate exercise
 - c. High elevations
 - d. Increased RBC destruction
3. Which of the following represents the most complete and correct listing of the most common clinical signs of anemia?
 - a. Fatigue, weakness, dyspnea, pallor
 - b. Urticaria, hypertension, inflammation, nausea
 - c. Nausea, hypertension, temperature elevation, melena
 - d. Rapid pulse, inflammation, temperature elevation, dehydration
4. What is the normal adult value for the reticulocyte count?
 - a. 1.0%–4.0%
 - b. 2.0%–6.0%
 - c. 11.5%–14.5%
 - d. 0.5%–2.0%
5. How is hematocrit measured on automated hematology instruments?
 - a. Centrifugation
 - b. Photometrically
 - c. Calculation (MCV \times RBC count) divided by the total sample volume
 - d. Calculation (MCH \times Hgb)

REVIEW QUESTIONS—cont'd

6. A patient has the following results: Hct 26%; Hgb 8 g/dL; and RBC count $3.5 \times 10^6/\mu\text{L}$. Calculate the RBC indices—MCV, MCH, and MCHC—and determine the classification of the anemia.
 - a. MCV 88 fL; MCH 30 pg; MCHC 33 g/dL; normocytic, normochromic
 - b. MCV 101 fL; MCH 33 pg; MCHC 35 g/dL; macrocytic, normochromic
 - c. MCV 74 fL; MCH 22 pg; MCHC 31 g/dL; microcytic, hypochromic
 - d. MCV 70 fL; MCH 22 pg; MCHC 38 g/dL; microcytic, hyperchromic
7. In a healthy individual, the body produces new RBCs to compensate for what approximate daily loss?
 - a. 0.01%
 - b. 0.1%
 - c. 10.0%
 - d. 1.0%
8. A microcytic hypochromic anemia can be seen on the peripheral blood smear in what type of anemia?
 - a. Iron-deficiency anemia
 - b. Megaloblastic anemia
 - c. Hemolytic anemia
 - d. Anemia of liver disease
9. Which of the following is considered in the interpretation of a bone marrow aspirate smear?
 - a. RBC indices
 - b. Type and amount of hemoglobin
 - c. M:E ratio
 - d. Reticulocyte count
10. What hormone produced in the kidney is usually increased in anemia?
 - a. Erythropoietin
 - b. Pyridoxine
 - c. Thiamin
 - d. Cytokines
11. Regarding the new RBC parameters and reticulocyte indices offered in the latest generation of hematology analyzers, the hemoglobin content of the reticulocyte allows for:
 - a. Early detection of iron-deficiency anemia
 - b. Accurate diagnosis of megaloblastic anemia
 - c. Measurement of stored iron
 - d. Measurement of RBC enzymes

See answers at the back of this book.

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Iron Metabolism and Hypochromic Anemias

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CHAPTER OUTLINE

Normal Iron Metabolism

- Distribution and Requirements
- Daily Iron Requirements
- Sources of Iron
- Iron Absorption and Transport
- Iron Regulation
- Iron Storage

Laboratory Evaluation

- Serum Iron
- Total Iron-Binding Capacity
- Transferrin Saturation
- Ferritin
- Transferrin Receptor
- Free Erythrocyte Protoporphyrin and Zinc Protoporphyrin
- Bone Marrow Iron
- Reticulocyte Count/ Reticulocyte Corpuscular Hemoglobin
- Hepcidin

Iron-Deficiency Anemia

- Etiology
- Pathophysiology
- Clinical Findings
- Laboratory Testing and Results
- Treatment

Anemia of Chronic Inflammation

- Etiology
- Pathophysiology
- Clinical Findings
- Laboratory Testing and Results
- Treatment

Sideroblastic Anemia

- Etiology
- Pathophysiology
- Clinical Findings
- Laboratory Testing and Results
- Treatment

The Porphyrrias

Iron Overload and Hemochromatosis

- Etiology
- Pathophysiology
- Clinical Findings
- Laboratory Testing and Results
- Treatment

Case Study 7-1

Case Study 7-2

Case Study 7-3

Case Study 7-4

Summary Chart

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 7-1** Explain the function of iron in relation to hemoglobin.
- 7-2** Evaluate the processes of iron absorption, transport, regulation, and storage related to erythropoiesis.
- 7-3** Identify the most common causes of hypochromic anemias given complete blood count (CBC) results.
- 7-4** Assess the pathophysiology of iron deficiency anemia, anemia of chronic inflammation, sideroblastic anemia, and the porphyrias.
- 7-5** Differentiate a ringed sideroblast from other abnormal cells.

- 7-6** Discuss the main cause of acquired sideroblastic anemia.
- 7-7** Differentiate iron deficiency anemia, anemia of chronic inflammation, and sideroblastic anemia based on laboratory results.
- 7-8** Define the porphyrias.
- 7-9** Analyze the pathophysiology of iron overload and hereditary hemochromatosis, and list common disorders associated with these disorders.
- 7-10** Evaluate laboratory results that indicate iron overload and hereditary hemochromatosis.

The microcytic hypochromic anemias are a group of red blood cell disorders that involve a defect in hemoglobin synthesis due to a deficiency of iron or abnormal iron utilization. These anemias include iron-deficiency anemia (IDA), anemia of chronic inflammation (ACI), and sideroblastic anemia. The most common of these is IDA, which affects more than 1.2 billion individuals worldwide.¹ Microcytic hypochromic anemias also include disorders of globin chain synthesis, which comprise the thalassemias. These globin chain defects are discussed in Chapter 12.

Erythropoiesis is a highly regulated process throughout the entire life span of each individual. Hemoglobin synthesis is an integral part of erythropoiesis and requires three compounds: iron, globin, and protoporphyrin. Each hemoglobin molecule consists of four heme groups and four globin chains. Each heme group contains a protoporphyrin ring plus an iron molecule. Adult hemoglobin A contains two alpha and two beta polypeptide chains. For a review of hemoglobin formation and function, refer to Chapter 2. This chapter reviews normal iron metabolism followed by a

discussion of IDA, ACI, sideroblastic anemia, and hereditary hemochromatosis.

Normal Iron Metabolism

Iron is an essential element for all living organisms. It is required by every cell in the body and is the essential component of the heme complex. It is important for cellular growth, oxygen transport, and the proliferation of red blood cells. Key factors in the metabolism of iron include distribution and requirements, daily requirements, absorption and transport, regulation, and iron storage.

Distribution and Requirements

The average adult has a total body iron content of between 3 and 5 g. Two-thirds of the total body iron is found in hemoglobin.² The remainder is found in myoglobin, iron-dependent metabolic proteins, and storage pools of iron, and a small amount is in circulation bound to transferrin.³ Iron storage pools are found in the bone marrow, liver, and spleen. The majority of this stored iron is in the form of ferritin or hemosiderin.² Ferritin iron is easily mobilized by the body for utilization, and serum ferritin levels are used as an indirect

measure of the iron stores. Hemosiderin, another form of storage iron, represents precipitated aggregates of ferritin and is less readily available for utilization.^{4,5} Iron metabolism and maintenance of body stores is a tightly regulated process. Daily iron intake, absorption, and losses are usually very small. Body iron is repeatedly recycled, and the small amount of iron that is lost each day is replaced by the diet. This small amount of iron lost each day through cellular shedding of enterocytes (intestinal mucosal cell) and sweating is approximately 1 mg.² The normal life span of the red blood cell (RBC) is approximately 120 days. Each day, 1% of red cells are taken out of circulation. As a result, 20–30 mg of iron are needed each day to replace the iron lost by senescent red blood cells. The majority of this iron comes from recycling, because most of the iron from RBC turnover is taken up by the mononuclear phagocytic system (RES cells) and reutilized.⁶ The daily iron turnover is illustrated in Figure 7-1.

Daily Iron Requirements

Only a fraction of dietary iron is absorbed. Of the recommended daily allowance (RDA) of 8 mg/day for males and 18 mg/day for females ages 19–50 years, the normal daily

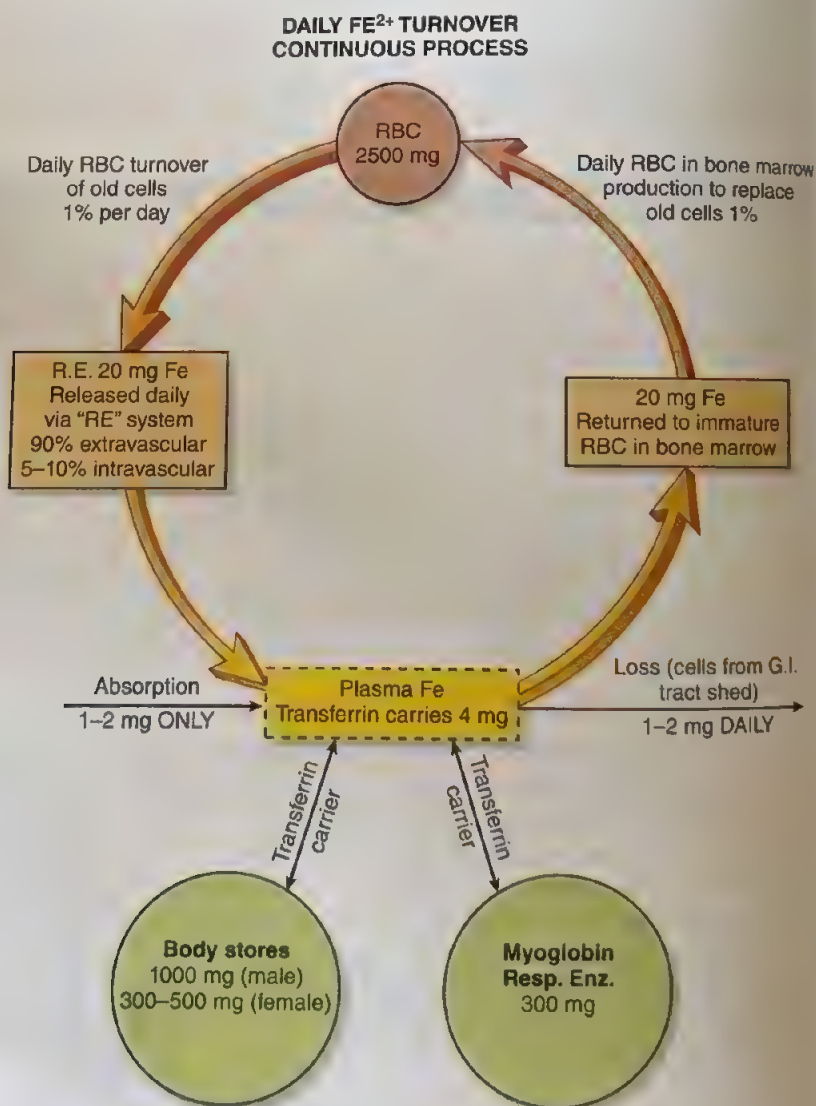


FIGURE 7-1 Daily iron turnover and body iron distribution.

absorption is 1–2 mg of iron from the diet.²⁷ Eighty percent of daily iron intake is directed to hemoglobin synthesis. Daily iron requirements are affected by a number of physiological states, including menstruation, pregnancy, and growth. Normally, during menstruation, women can lose approximately 1 mg of iron per day, in addition to the 1–2 mg typically lost daily.⁵

ADVANCED CONTENT

The extent of menstrual bleeding is extremely variable, and some women may lose 10–40 mg of iron a month as a result of menstruation.⁹ For pregnant women, the recommended daily allowance (RDA) for iron increases from 18 mg to 27 mg. The minimum daily requirements (MDR) are not consistent throughout the pregnancy. The MDR in the first trimester are actually lower than prepregnancy because the woman is no longer menstruating. By the third trimester, the daily requirement of iron can increase to 3.0–7.5 mg.¹⁰ At delivery, a blood loss of more than 500 mL for a vaginal birth and 1,000 mL for a caesarean is considered a postpartum hemorrhage (PPH).¹¹ In the past, it has been noted that lactation increases iron demand, but recent data shows that the RDA for lactating mothers is actually less than other adult females. According to the National Institutes of Health, the RDA of iron for females between the ages of 19–50 years is 18 mg. For lactating females, it is 10 mg. This is likely due to the fact that many nursing mothers have lactational amenorrhea, and breast milk is very low in iron. It should be noted that a postpartum woman may be iron deficient due to increased demands during pregnancy and blood loss during delivery.^{7,12,13}

During periods of growth, such as infancy and adolescence, iron requirements are substantially increased. Because milk is a poor source of iron, many baby formulas are supplemented with iron. Infants who are fed only mothers' breast milk for longer than 4 months are at a significant risk of developing IDA.^{1,7}

Sources of Iron

Dietary iron comes from heme (animal) and nonheme (plant and animal) sources. Many foods are rich in iron, including meats, seafood, legumes, green vegetables, fortified cereals, and prunes (Table 7-1). The bioavailability of iron ingested in the diet is only 5% to 18%.⁷ The foods that increase and decrease iron absorption are listed in Table 7-2.

Iron Absorption and Transport

Absorption of heme iron is not well understood, but it is thought to involve a heme carrier on the membrane of the proximal intestine. Once inside the enterocyte, it is likely a heme oxygenase releases ferrous iron, and it undergoes the same common process as nonheme iron as it leaves the enterocyte.² Regulation of nonheme iron absorption occurs within the intestinal mucosa of the small bowel. The vast majority of iron is absorbed in the duodenum and the first portion of the jejunum. Iron molecules within the diet and

TABLE 7-1 Iron-Containing Foods

Foods High in Iron	Foods Moderate in Iron
Organ meats	Muscle meats (beef, fish, fowl)
Wheat germ	Prunes
Brewer's yeast	Cereals
Legumes	Green vegetables
Whole grain breads	Shrimp
Oatmeal	Oysters

TABLE 7-2 Substances That Increase and Decrease the Absorption of Iron

Increased	Decreased
Ingestion of acidic foods	Phosphates
Ascorbic acid (vitamin C)	Phytates
	Foods that form insoluble iron complexes

iron complexes within the body can be present in various iron states (Table 7-3). Ferric iron (Fe^{3+}) is the most common dietary form of iron. The acidic pH (<4) of gastric secretions and the presence of reducing substances such as ascorbic acid in the stomach help reduce ferric iron (Fe^{3+}) to the ferrous form (Fe^{2+}) by one of several reductase enzymes such as Duodenal Cytochrome B (DCYTB). The strong acidic environment of the stomach, in conjunction with pancreatic enzymes, facilitates optimal absorption in the duodenum and jejunum.

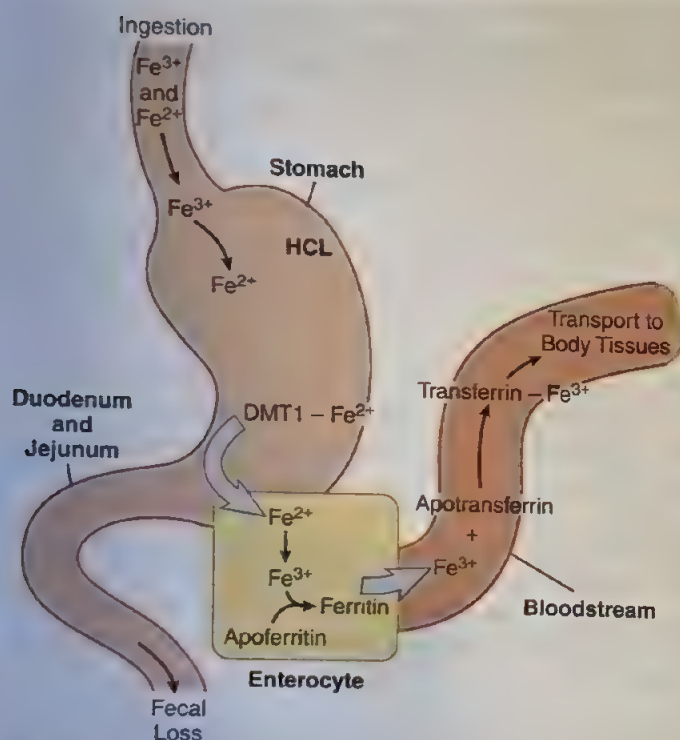
ADVANCED CONTENT

Ferrous iron ($\text{Fe} + 2$) is carried from the intestinal lumen into the enterocytes of the duodenum and jejunum by **Divalent Metal Transporter 1 (DMT1)**. If the body does not have an immediate need of iron, it is retained within the enterocytes as the iron storage form, ferritin (apoferritin + ferric iron). If required by the body, the ferrous iron is exported by **ferroportin 1 (FPN1)** across the enterocyte basolateral membrane. This process is facilitated by oxidation of the iron to $\text{Fe} + 3$ by a copper-dependent enzyme, **hephaestin**. Ferroportin is also important in the recycling of iron by moving iron out of the macrophages of various organs, such as the bone marrow, into the bloodstream to bind transferrin.

In plasma, the ferric iron circulates tightly bound to **transferrin** (apotransferrin + ferric iron). Transferrin assists iron delivery to the erythroblasts in the bone marrow and to storage in the bone marrow, liver, and spleen. The **chelation** of iron by transferrin binding maintains iron in a soluble form. Two atoms of ferric iron can bind to one transferrin molecule (Fig. 7-2).

TABLE 7-3 Iron (Fe) Molecules and Compounds

Fe Molecule/Compound	Fe State	Location	Function
Dietary Fe	Fe^{2+} or Fe^{3+}	Upper GI tract	Hemoglobin, myoglobin, enzyme synthesis
Hemoglobin	Fe^{2+}	RBCs	Bind oxygen
Transferrin	Fe^{3+}	Serum	Transport Fe
Ferritin	Fe^{3+}	Serum and tissue sites	Fe storage
Hemosiderin	Fe^{3+}	Bone marrow and other tissue sites	Fe storage

IRON (Fe) ABSORPTION AND TRANSPORT**FIGURE 7-2** Schematic of iron intake and absorption through the mucosal cells in the small intestine. Absorbed iron is converted to ferritin for storage or transported bound to transferrin for distribution to body tissues.

Transferrin bound iron is moved into the nucleated red blood cell (NRBC) and reticulocytes through the **transferrin receptor 1 (TfR1)** located on the surface membrane of the red cell. The endosome (membrane-bound cytoplasmic vesicle) that is created is acidified allowing the iron to be released and reduced to the ferrous form. The iron transporter, divalent metal transport 1 (DMT1), moves the iron into the RBC cytoplasm. The process by which iron enters the mitochondria is unclear.¹⁴ It has been demonstrated that there is some direct transfer of iron between the endosome and the mitochondria.¹⁵ In the final step of hemoglobin synthesis, the insertion of iron into protoporphyrin IX is catalyzed by **ferrochelatase**.¹⁶ Any excess iron not needed for hemoglobin synthesis is stored as ferritin¹⁴ (see Chapter 2).

Despite the large amount of iron in the Western diet, iron deficiency continues to be a significant cause of morbidity

in North America and throughout the world. Many food products, including flour and baby formulas, are now supplemented with iron to help alleviate the problem.⁷ Factors that affect the absorption of iron include:

- Amount and type of iron accessible from food
- Functional state of gastrointestinal mucosa and pancreas
- Current iron stores
- Erythropoietic needs

Iron Regulation

The rate at which iron is transferred from the enterocyte to the bloodstream is regulated by the body's iron levels and requirements.

ADVANCED CONTENT

Ferroportin transports iron not only from enterocytes but also from macrophages and hepatocytes into the bloodstream. **Hepcidin**, a protein produced in the liver, is the key regulator in this process. Hepcidin binds to ferroportin, inactivating it and thus decreasing the iron released from cells such as macrophages and enterocytes.¹⁴

Regulation of hepcidin is multifaceted. A complex pathway has been identified but is not fully understood. The process involves the bone morphogenetic protein (BMP)-SMAD signaling complex, hemojuvelin (HJV), transferrin receptors 1 and 2 (TfR1 and TfR2), and matriptase-2, also known as transmembrane serine protease 6 (TMPRSS6). The BMP-SMAD signaling pathway and HJV play major roles in the expression of hepcidin in the liver and systemic iron homeostasis. TMPRSS6 regulates this process.^{1,8}

The **hemochromatosis** gene produces a HFE protein that binds to transferrin receptor 1 (TfR1). The HFE protein interacts with TfR1 to control iron uptake by regulating the production of hepcidin in the liver. When iron levels are decreased, HFE protein can bind to TfR1 and is not available to produce hepcidin. Thus, ferroportin is not inactivated, so more iron is absorbed and released from cellular storage. When there is plenty of iron, the HFE protein does not bind as readily to the TfR1 and is available to produce hepcidin, thus blocking the release of iron from cellular storage.¹⁷ A mutation in the HFE gene causes hereditary hemochromatosis (see Hereditary Hemochromatosis later in this chapter).

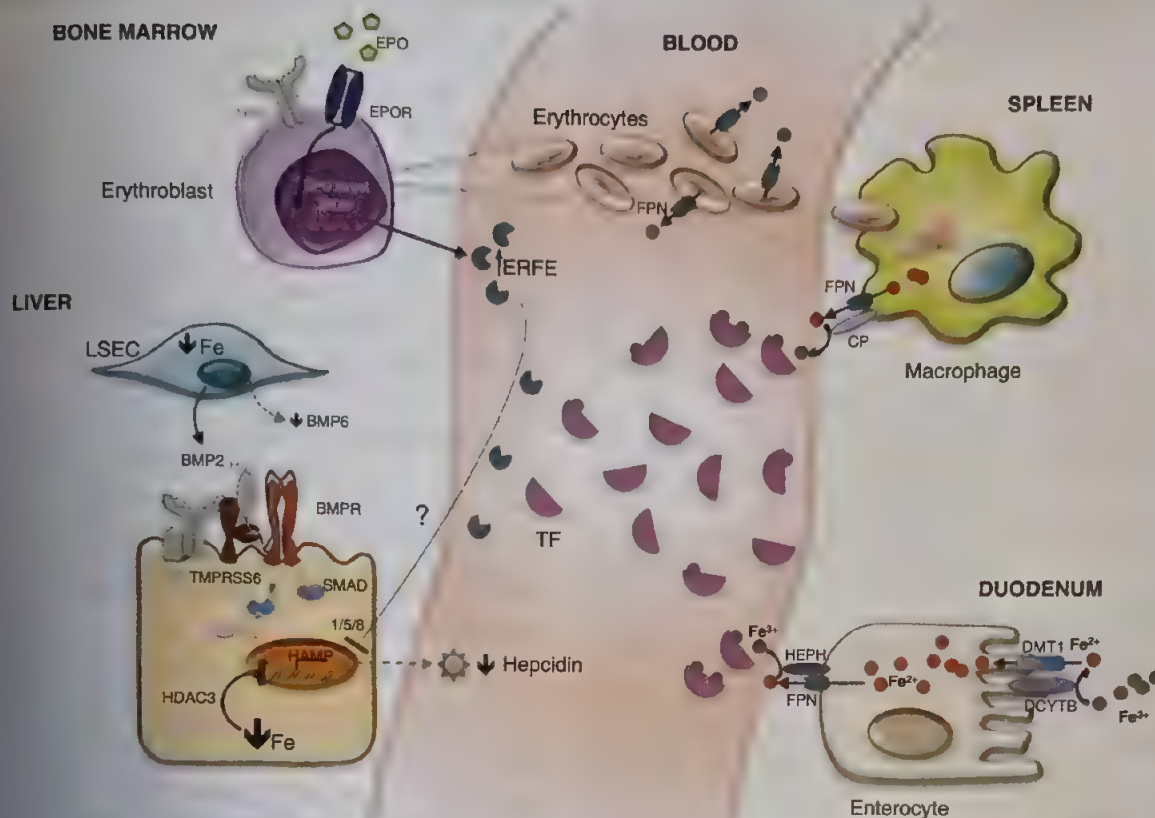


FIGURE 7-3 Mechanisms of hepcidin inhibition in iron deficiency anemia. In the hepatocytes, bone morphogenic protein (BMP)-SMAD signaling, the main activator of hepcidin, is low because low levels of BMP6 are produced by liver sinusoidal endothelial cells (L-SEC), the BMP coreceptor hemojuvelin (HJV) is cleaved from the hepatocyte surface by the transmembrane serine protease 6 (TMPS6), and the second transferrin receptor (TFR2) is not stabilized on the cell surface in the absence of the ligand diferric transferrin (TF). Low hepcidin levels increase iron absorption by enterocytes and recycling by macrophages through increased activity of the iron exporter FPN. In mild iron deficiency in the absence of hypoxia, increased EPO sensitivity is due to the loss of TFR2 on erythroblast surfaces. In iron deficiency anemia, hypoxia increases EPO. Increased ERFE fully blocks the hepcidin pathway, although the molecular mechanism of hepcidin inhibition by ERFE remains unknown (?). BMPR, BMP receptor; DCYTB, duodenal cytochrome B; DMT1, divalent metal transporter 1; EPOR, EPO receptor; HEPH, hephestin. (Adapted from Camaschella C. Iron deficiency. *Blood*. 2019 Jan 3;133(1):30-39. doi: 10.1182/blood-2018-05-815944. Epub 2018 Nov 6. PubMed PMID: 30401704.)

When iron is needed for erythropoiesis, erythropoietin (EPO) not only stimulates the formation of new red blood cells, it enhances a hormone produced by erythroblasts, **erythroferrone (ERFE)**, that suppresses hepcidin.¹⁸ The mechanisms of hepcidin inhibition in iron deficiency are demonstrated in Figure 7-3.

Iron Storage

Senescent red cells are engulfed by macrophages and recycled. Iron that is not used for erythropoiesis is stored in the mononuclear phagocytic system (MPS) or reticuloendothelial (RE) cells of the bone marrow, liver, and spleen. Iron taken in excess is stored in two forms, ferritin and hemosiderin. RE cells ingest old red cells and catabolize the hemoglobin to recycle the iron.⁶ Free iron is toxic; therefore, it must be sequestered by a protein. The major intracellular form of storage iron is ferritin (apoferritin + ferric iron).

Ferritin is water soluble and is easily mobilized by the body for utilization.

Transferrin receptor 1 (TfR1) on the surface of the cell is capable of binding two molecules of transferrin, each carrying one or two molecules of iron. The iron content of the cell is regulated by control of the iron uptake and storage capacity. As cellular iron levels fall, the levels of ferritin decrease, and the transferrin receptors increase. When cellular iron increases, ferritin increases, and TfRs fall.¹⁴

The second storage form of iron is a degradation product of ferritin, hemosiderin. Hemosiderin is not water soluble. The iron in hemosiderin is released more slowly than that from ferritin and is less readily available for utilization. Hemosiderin represents aggregates of iron and can be visualized in tissue with the use of a Prussian blue stain for iron. Hemosiderin appears as granules and aggregates. Hemosiderin does become available in iron-deficient patients.^{5,19,20} Proteins involved in iron metabolism are summarized in Table 7-4.

TABLE 7-4 Proteins Involved in Iron Metabolism

Protein	Function
Transferrin	Iron-binding transport protein in plasma and extracellular fluid with two iron binding sites
Transferrin receptor	Receptor mediated ferric transferrin with two transferrin binding sites
Ferritin	Iron storage
Hemosiderin	Iron storage (less readily available)
Hemochromatosis Gene Protein (HFE)	HFE binds with transferrin receptor reducing the affinity for transferrin
Divalent Metal Transporter 1 (DMT1)	Iron transport protein from gastrointestinal lumen into the duodenal enterocyte; from erythroblast endosome to cytoplasm
Ferroportin 1	Transports iron from enterocyte and macrophages into macrophages into bloodstream
Hephaestin	Copper dependent enzyme that oxidizes iron to facilitate its transport by ferroportin across enterocyte membrane into bloodstream
Hepcidin	Inactivates ferroportin thus decreasing iron absorbed and released from cells
Erythroferrone (ERFE)	Hormone produced by erythroblasts that suppresses hepcidin

CRITICAL THINKING QUESTION

7-1 Erythropoiesis and hemoglobin production will be affected by altered iron status. What parameter(s) of the CBC can demonstrate an early response to therapy? How might an individual who underwent a bowel resection be affected hematologically?

See answers to all Critical Thinking Questions at the back of this book.

Laboratory Evaluation

The routine laboratory assessment of iron includes the measurement of serum iron, total iron-binding capacity (TIBC), transferrin, percent saturation of transferrin, serum transferrin receptors,

and ferritin. Zinc protoporphyrin (ZPP) is an indirect assessment of iron availability. In more complex cases, bone marrow iron, hepcidin levels, and reticulocyte data may be evaluated.² Reference ranges for iron status are listed in Table 7-5.

Serum Iron

Serum iron is a measure of transferrin-bound iron. Normal serum iron concentration for males is 65 to 170 mcg/dL. In women it is usually lower. The concentration of iron fluctuates in individuals and should not be used for investigation of iron metabolism without other laboratory tests.^{22,23} Traditionally, early morning specimens were preferred because of diurnal variation. The clinical significance of this effect has been questioned.²⁴

Total Iron-Binding Capacity

Total iron-binding capacity (TIBC) is the total amount of iron that can be bound by transferrin in the plasma or serum. Values increase in IDA and decrease in iron overload. Each gram of transferrin will bind 1.4 mg of iron. The normal range is 250 to 350 mcg/dL. The binding capacity is normally about one-third saturated.

Transferrin Saturation

Percent saturation of transferrin is functionally measured as the maximum amount of iron that is bound in plasma or serum. Serum iron and TIBC are used to calculate the percent saturation. A transferrin saturation value below 16% may be an indicator of iron deficiency. Other disorders such as ACL and pregnancy may result in low transferrin saturation without IDA. Transferrin saturation is consistently increased in iron overload.^{1,23} The percent saturation is calculated as follows:

$$\% \text{ saturation} = \frac{\text{serum iron}}{\text{TIBC}} \times 100\%$$

Ferritin

Serum ferritin is directly proportional to the amount of iron stored as ferritin. Ferritin is a much better measurement than serum iron and TIBC for the assessment of body iron stores. Normal range for ferritin is 24 to 336 mcg/L for men and 11 to 307 mcg/L for women. When low, ferritin is a good index of iron depletion. It is increased in iron overload. Ferritin is an acute phase reactant protein and is increased in inflammatory states, malignancy, and infections, as well as liver disease.³¹

Transferrin Receptor

Measurement of serum transferrin receptors (sTfRs) is another tool for the assessment of iron status. The sTfRs are inversely

TABLE 7-5 Iron Status: Reference Ranges*

	Serum Iron mcg/dL	TIBC mcg/dL	Transferrin mcg/dL	%Transferrin Saturation	Ferritin mcg/L
Adult male	65–170	250–450	200–400	20–55	20–250
Adult female	50–170	250–450	200–400	15–50	10–120
Newborn	40–250	100–400	130–275	12–50	50–600
Adolescent	50–150	250–450	200–400	15–55	10–150

*Please note that reference ranges vary by institution, patient population, and testing methodology.

proportional to the amount of body iron. The concentration is increased in iron deficiency but only when the iron stores are depleted. The reason is that, with the lack of intracellular iron, the regulatory proteins directly increase the synthesis of transferrin receptors. sTfRs do not increase with anemia of chronic inflammation. sTfRs assay is not widely used.

Free Erythrocyte Protoporphyrin and Zinc Protoporphyrin

Free erythrocyte protoporphyrin (FEP) is a heme precursor into which iron is incorporated to form the heme molecule. When iron is not available to be incorporated into this protoporphyrin ring, excess protoporphyrins form. These excess rings complex with zinc to form zinc protoporphyrin (ZPP). ZPP is increased in iron-deficient erythropoiesis. The ZPP value correlates inversely with the ferritin level.

Bone Marrow Iron

While Prussian blue staining of bone marrow iron stores may be helpful, it is rarely justified unless investigating a possible sideroblastic anemia, myelodysplastic syndrome, or other complex case.^{25,26}

Reticulocyte Count and Reticulocyte Corpuscular Hemoglobin (CHr)

Reticulocytes numbers will be decreased in cases of diminished and ineffective erythropoiesis.²⁷ Decreased reticulocyte corpuscular hemoglobin (CHr), a data point now available on many hematology analyzers, is an early indicator of iron-deficient erythropoiesis. Monitoring this value is also a good indicator of a patient's response to iron therapy.²⁸

Hepcidin

Hepcidin assays are available, but its utility in adding value to the current iron studies is still to be determined.²¹ It may be used for more complex cases such as coexisting conditions of iron deficiency anemia and anemia of chronic inflammation.²⁷

Iron-Deficiency Anemia

Iron-deficiency anemia (IDA) is the most common cause of anemia worldwide, with more than 1.2 billion people affected. In the United States, approximately 2% of men and 5% of women are affected by IDA.^{1,29}

Iron deficiency is described as a microcytic hypochromic anemia. The red blood cells are small in size and have an increase in central pallor, since they are lacking hemoglobin. Iron deficiency without anemia is even more common. IDA results when there is a lack of adequate iron stores in the body to meet physiological needs for the production of red blood cells. Other issues related to IDA include diminished cognitive function, decreased physical ability, and unfavorable pregnancy outcomes.

Etiology

IDA may occur because there is an increased demand for iron, abnormal utilization of iron, poor diet, increased blood loss, or malabsorption. IDA can develop slowly after the normal stores of iron have been depleted or may occur more rapidly when there is an increased demand for iron. Individuals most at risk of developing IDA due to increased iron demand

include infants, preschool children, adolescents, menstruating women, and pregnant women.¹ For high-risk groups, see Box 7-1. Causes of IDA are summarized in Box 7-2.

Diet and Increased Need

Nutritional deficiency occurs when insufficient iron taken in through the diet does not meet the need for erythropoiesis. If the diet has inadequate iron, the body stores of iron will continue to deplete. Infants, children, and adolescents are at a higher risk for developing IDA because of higher iron requirements needed during growth spurts.²² The iron absorption requirement on average is 1 mg per day to support normal growth and replace normal loss. Toddlers have increased iron needs because cow's milk is low in iron. As mentioned previously, pregnancy also makes demands on the mother's body to provide iron for her needs and iron for the developing fetus.

Blood Loss

Excessive loss of iron from the body through blood loss can lead to IDA. IDA in adult males is usually caused by chronic blood loss from the gastrointestinal tract (GI). Blood loss from the GI tract includes occult bleeding, peptic ulcers, tumors, malignancies, hemorrhoids, and hiatal hernia. Typically, blood loss from the GI tract occurs in elderly patients. In women, IDA occurs via blood loss through menstruation. When the red blood cells are lost outside of the body, the iron cannot be recycled for reuse.

BOX 7-1 High-Risk Groups at High Risk for Iron-Deficiency Anemia

Prenatal/Neonatal

- Premature birth
- Low birth weight
- Anemia during pregnancy
- Low iron formula
- Lack of iron supplements after 6 months in breastfed infants

Infancy/Childhood

- Restricted diets
- Lack of iron supplements
- Growth spurts
- Chronic infection
- Chronic or acute blood loss

Adolescent/Adult

- Fad diets
- Menstruation
- Excessive weight gain
- Pregnancy
- Lactation and breastfeeding
- Elderly
- Improper diet
- Chronic bleeding
- Gastrointestinal bleeding

Social/Economic

- Low socioeconomic groups
- Recent immigration from developing countries

BOX 7-2 Causes of Iron-Deficiency Anemia**Inadequate Absorption**

- Inflammatory bowel disease
- Resection of the bowel
- Celiac disease
- Antacid therapy
- High gastric pH
- Loss or dysfunction of absorptive enterocytes
- Poor bioavailability
- Excess bran

Decreased Iron Intake

- Meat-poor diet
- Cereal-rich diet
- Malabsorption
- Elderly

Increased Iron Utilization

- Growth spurts
- Pregnancy

Loss of Iron

- Gastrointestinal blood loss
- Ulcer
- Gastritis
- Epistaxis
- Hemorrhoids
- Menorrhagia
- Pulmonary blood loss
- Malignancy or cancer
- Trauma
- Excess phlebotomy

Malabsorption

Malabsorption is an uncommon cause of IDA. If iron absorption is impaired owing to the absence of gastric acids, which helps reduce dietary iron from ferric to the ferrous form, an IDA can develop. This can also be seen with patients suffering from malabsorption syndromes such as gastrectomy, gastric bypass, celiac disease, atrophic gastritis, or any disease that will compromise intestinal absorption.⁷

Pathophysiology

IDA develops in the following stages over a period of time: stage 1, iron depletion; stage 2, iron-deficient erythropoiesis; and stage 3, the final stage, development of IDA. The stages of IDA are reviewed and summarized in Table 7-6.

Stage 1: Iron Depletion

Stage 1 is generally asymptomatic. The iron stores in the bone marrow are depleted without a decrease in serum iron. The hemosiderin content of the cells of the RES in a bone marrow aspirate is decreased or absent when stained with Prussian blue (Fig. 7-4). As a result, serum ferritin (a measure of stored iron) is decreased while mucosal absorption is increased. In an attempt to compensate, the liver will synthesize more transferrin, producing an increase in TIBC. An anemia may not be evident (complete blood count [CBC] is normal), and the RBC morphology is normal. This stage creates no overt effect on erythropoiesis. The red cell distribution width (RDW), however, is usually increased, which is sometimes the first indication of an anemia developing.^{7,30}

Stage 2: Iron-Deficient Erythropoiesis

The second stage is iron-deficient erythropoiesis and is sub-clinical. Plasma iron levels drop. When there is a decrease in

TABLE 7-6 Stages of Iron-Deficiency Anemia

Laboratory Features	Normal*	Stage 1: Iron Depletion (IDA Without Anemia)	Stage 2: Iron Deficient Erythropoiesis (IDA With Mild Anemia)	Stage 3: IDA (Severe Anemia)
Serum iron level	65–170 mcg/dL	Normal to decreased	Decreased	Decreased
Total iron binding capacity	250–450 mcg/dL	Normal to increased	Increased	Increased
Serum ferritin level	12–300 mcg/L	Decreased	Decreased	Decreased
Percent transferrin saturation	M: 20%–50% F: 15%–50%	Normal	<15%	Decreased
TfR	1.5–2.75 mg/L	Normal	Increased	Increased
Zinc protoporphyrin (free erythrocyte protoporphyrin)	16–65 mcg/dL	Normal to increased	Increased	Increased
Hemoglobin	13–15 g/dL	Normal	Normal to decreased	Very decreased
Hypochromia	Not present	Not present	Slight	Marked
Mikrocytes	Not present	Not present	Slight	Present
Stainable bone marrow iron	Present	Absent or decreased	Absent	Absent

TfR = transferrin receptor levels; ID = iron deficiency; M = male; F = female.

*Please note that normal values vary by institution, patient population, and testing methodology.

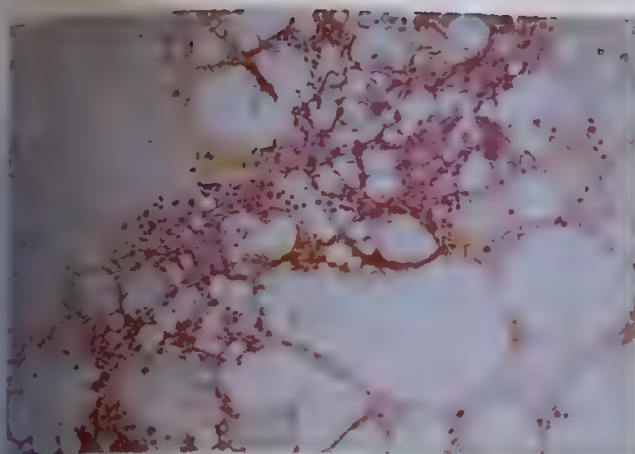


FIGURE 7-4 Bone marrow aspirate from a patient with iron-deficiency anemia stained with Prussian blue. Note the negative staining indicated by a lack of any blue stain indicating an absence of iron.

iron that is needed for heme synthesis, excess protoporphyrins accumulate and complex with zinc to form zinc protoporphyrins (ZPP). The hemoglobin and hematocrit values are usually normal and the red blood cells may begin to appear microcytic. Ferritin levels are decreased, TIBC increases, and TfRs increase on the surface of the red blood cells. A decrease in reticulocyte hemoglobin content (CHr) is an early indicator of iron-deficient erythropoiesis. Other iron-dependent tissues may be affected.^{7,14,28,31}

Stage 3: Iron-Deficiency Anemia

The final stage of anemia develops when the red blood cells are severely deficient in iron. The advanced stage will show a marked decrease in hemoglobin and hematocrit. Hemoglobin formation is delayed with the formation of red cells that are hypochromic and microcytic³¹ (Fig. 7-5). EPO levels increase, and there is ineffective erythropoiesis as a result of depleted storage iron and diminished transport of iron.²¹ The bone marrow shows decreased hemoglobinization and ragged

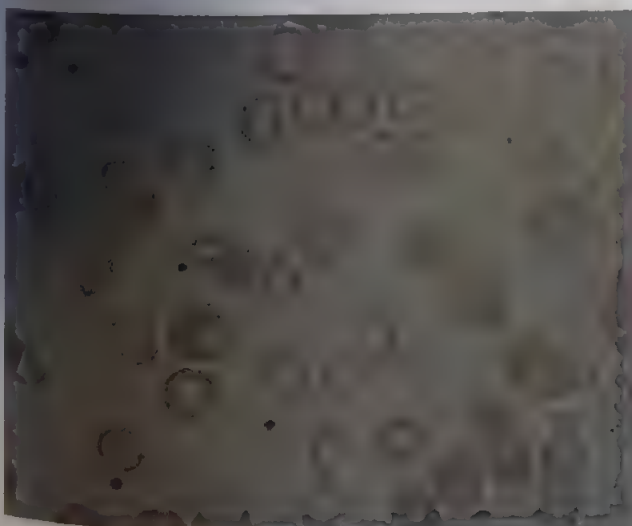


FIGURE 7-5 Iron-deficiency anemia characterized by microcytic, hypochromic red blood cells.

cytoplasm in red blood cell precursors³² (Fig. 7-6). At this stage, there is a severe deficiency in total body iron. Most patients are not diagnosed until this stage of the disease.

Clinical Findings

IDA may occur over a period of months to years. The clinical findings of IDA are varied. As the anemia develops in severity and duration, the clinical symptoms increase. Typical symptoms of IDA include fatigue; irritability; headache; weakness, especially with exercise; shortness of breath; tachycardia; and pale skin color or pallor. Other symptoms of a severe IDA are koilonychia (spooning of the nails) (Fig. 7-7), cheilitis (lip inflammation), glossitis (sore tongue), and muscle dysfunction (including the heart) (Fig. 7-8).

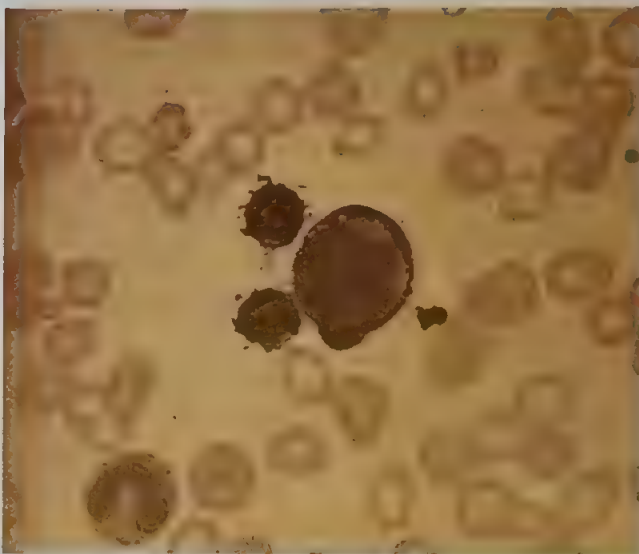


FIGURE 7-6 Bone marrow aspirate in iron-deficiency anemia showing ineffective erythropoiesis, "ragged" erythroid precursors. (From Bell A. Hematology. In Listen, Look and Learn. Bethesda, MD: Health and Education Resources, Inc., with permission.)



FIGURE 7-7 Koilonychia or spooning of the nails, characteristic of iron-deficiency anemia.

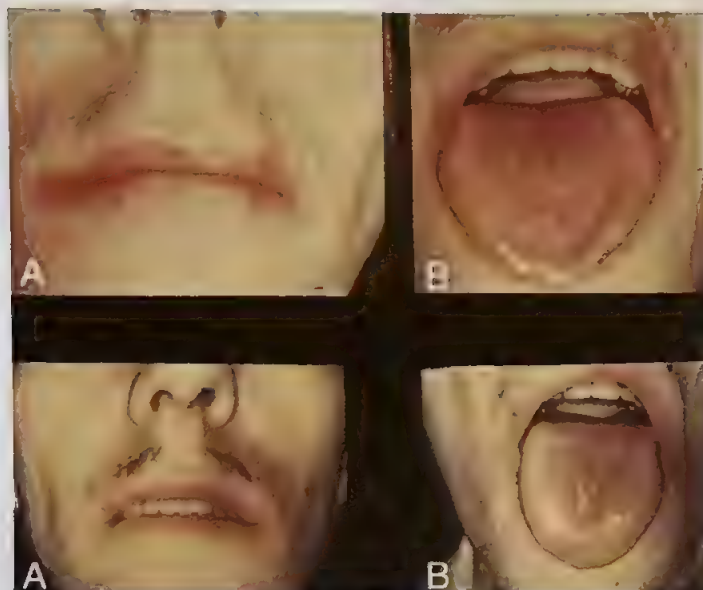


FIGURE 7-8 Clinical manifestations of iron-deficiency anemia. **A.** Cheilitis, before (top) and after (bottom) therapy. **B.** Glossitis before (top) and after (bottom) therapy.

Another manifestation that may be associated with IDA is pica. Pica is defined as the persistent eating or craving of nonfood substances such as clay, dirt (geophagia), or ice (pagophagia).

Infants with IDA are at risk for developmental, behavior, and motor difficulties. Symptoms include irritability, loss of memory, and difficulties in learning. They also have increased susceptibility to infection, poor growth, and even heart issues.³³

Laboratory Testing and Results

The features and indices associated with iron-deficiency anemia are summarized in Box 7-3.

Peripheral Blood

In patients with severe IDA, a microcytic hypochromic anemia is seen on the peripheral blood smear (see Fig. 7-5). The mean corpuscular volume (MCV), the mean corpuscular hemoglobin concentration (MCHC), and the mean corpuscular hemoglobin (MCH) will all be decreased. The value will be determined by the degree of anemia present. Microcytes, anisocytosis, and an increased RDW are the first morphological signs. The peripheral blood smear usually contains increased poikilocytosis with the presence of a few codocytes (target cells), elliptocytes or ovalocytes, and dacryocytes (tear-drop cells) in addition to microcytes. The reticulocyte count, which measures the ability for the bone marrow to produce new red blood cells, is decreased in relation to the severity of the anemia as a result of ineffective erythropoiesis.^{31,34} The white blood cell count may be low. The platelet count may be normal, increased, or decreased.³⁵

Iron Studies

Serum iron is decreased as a result of the depletion of iron stores, TIBC is increased, percent transferrin saturation is decreased, and TfRs are increased. Serum ferritin, a storage form of iron, is decreased in all stages of IDA and is usually

BOX 7-3 Indices and Features of Iron-Deficiency Anemia

Clinical

- Clinical findings depend on the severity of the anemia
- Severe anemias may be associated with pallor, weakness, and dyspnea

Morphological

- Usually hypochromic, microcytic RBCs
- Mild to moderate anisopoikilocytosis
- Decreased storage iron
- Decreased sideroblasts
- Absence of ringed sideroblasts

Laboratory

- Decreased serum iron
- Decreased serum ferritin
- Decreased % transferrin saturation
- Increased total iron-binding capacity (TIBC)
- Increased free erythrocyte protoporphyrin (FEP) and zinc protoporphyrin (ZPP)
- Increased serum soluble transferrin receptor levels
- Increased erythropoietin (EPO)
- Decreased reticulocyte corpuscular hemoglobin (CHr)

the first indication of an IDA developing. Serum ferritin is a sensitive marker of iron storage and is an important laboratory test to help differentiate IDA from other microcytic hypochromic anemias. The sTfR is useful in detecting IDA as these patients have an increased sTfR.^{1,34}

Bone Marrow

Usually a bone marrow assessment is not indicated for an uncomplicated case of IDA. The bone marrow shows a mild to moderate erythroid hyperplasia with a decreased M:E ratio and dyserythropoiesis results. A significant number of cells are destroyed as reticulocytes resulting in an erythroid hyperplasia that cannot compensate for the degree of anemia.^{36,37}

Treatment

The first choice in the treatment for IDA is correction of the primary disease state. The next step is oral dietary supplements, which are necessary to replenish the body stores.

ADVANCED CONTENT

Oral supplements of ferrous sulfate are the standard treatment.^{7,12} A major obstacle with oral supplements is the side effect of nausea and stomach discomfort. Other oral supplements including ferrous gluconate and ferrous fumarate can be used. In some cases where intestinal absorption of iron is impaired, intravenous administration of iron is used, which may also be given for patients who cannot tolerate iron supplements.

Blood transfusions are used only if the hemoglobin drops to dangerously low levels. Each hospital will determine its

own critical level, but generally red cell transfusion should only be used in cases of debilitating symptoms or risk of cardiovascular collapse. After therapy, reticulocytes begin to rise within 4 to 5 days. Hemoglobin levels will begin to increase in about 2 weeks. Restoration of the patient's iron stores usually takes about 6 months³ (Fig. 7-9).

CRITICAL THINKING QUESTION

7-2 Why is an increase in TIBC seen in the earliest stage of IDA, even before ferritin levels decrease?

Anemia of Chronic Inflammation

Anemia of chronic inflammation (ACI) or anemia of chronic disease is a common hematological disorder. ACI is the second-most prevalent anemia after IDA and the most commonly found anemia in hospitalized patients or those with chronic disease processes. It is commonly seen in elderly patients, but it can be observed in patients of any age.

Etiology

ACI is complex in nature and is associated with chronic infections, autoimmune disease, chronic kidney disease, malignant neoplasms, and other conditions associated with chronic inflammation^{27,38} (Box 7-4). ACI is considered an anemia of disordered iron metabolism because the iron stores are high and mobilization of the iron is impaired.

Pathophysiology

Much has been learned about the pathogenesis of ACI during the last decade. Three major mechanisms have been described that act through mediators of an activated immune system to cause anemia.

The first cause is a response of iron restriction to many inflammatory cytokines released. Hepcidin is a hormone produced by hepatocytes to regulate iron levels, but it is also an acute phase reactant. Thus, increased levels of hepcidin result in the increased inactivation of ferroportin. Under normal conditions, ferroportin exports and recycles iron in the blood. This reaction to chronic inflammation inhibits the absorption

BOX 7-4 Disorders Associated With Anemia of Chronic Inflammation (ACI)

Infections

- Tuberculosis
- Chronic osteomyelitis
- Fungal infections

Neoplasms

- Carcinomas
- Malignant lymphoma
- Multiple myeloma

Autoimmune Disorders

- Systemic lupus erythematosus (SLE)
- Rheumatoid arthritis
- Sarcoidosis

of iron from the duodenum and blocks the release of iron stores from macrophages, causing decreased iron available for hemoglobin synthesis.

ADVANCED CONTENT

Inflammation is a component of innate immunity and is regulated by the interleukin-6 (IL-6) family of cytokines.³ IL-6 interacts with the complement system and pattern recognition receptors (i.e., TLR4) and modulates the adaptive immune response for defense against pathogens (bacteria, viruses, etc.). Because of its role in initiation of inflammation and the acute phase response, IL-6 has a big role in chronic disease and autoimmunity. Additionally, IL-6 is an adipokine or cytokine secreted by fat tissue that has a role in the increased inflammation seen in obesity.³

Inflammation is one of the body's responses to tissue injury. Inflammatory and hemostatic responses occur simultaneously to control any damage at the injured area. The coagulation cascade and the complement, fibrinolytic, and kinin systems also interact to modulate inflammation.

Complement can be activated by the antibody-induced classical pathway or directly by microorganisms via the alternative pathway. The presence of C3a, C5a, and other chemotaxins attracts phagocytes to the site of injury, where they recognize and phagocytose foreign substances or organisms. Neutrophils, monocytes, and macrophages possess receptors for complement that can induce both exocytosis of granules (containing proteolytic enzymes, free ion radicals, and other inflammatory metabolites) and endocytosis of complement-coated foreign substances.

Inflammation will continue as long as injury and damage continue. When the source of inflammation is persistent, mediators from the humoral and cell-mediated immune responses contribute to the onset of anemia (Fig. 7-10). The cytokines that contribute to inflammation are outlined in Table 7-7, along with their target cells and activities.

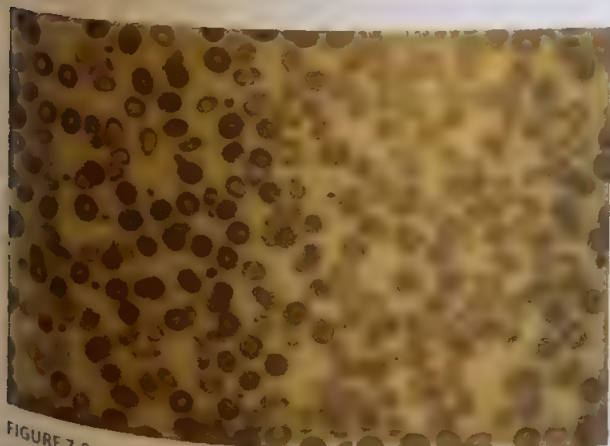


FIGURE 7-9 Peripheral blood of a patient with iron-deficiency anemia after therapy. Note the two populations of red blood cells: microcytic and normal cells.

FIGURE 7-10 Mechanism of humoral and cellular immunity. **A.** The antigen phagocytized by the APC is digested, and small antigenic fragments or epitopes are associated with the class II MHC and presented to a T cell with a receptor specific for the antigen. Formation of the antigen-receptor complex between the two cells and IL-1 secreted by the APC provide the signals for the T cell to be activated and secrete IL-2 for its autostimulation and proliferation to effector T-helper cells. **B.** Humoral immunity. The T-helper effector cell (CD4) and some of its lymphokines provide the necessary signals for the B cell with the same antigen specificity to be activated and proliferate to B memory and antibody-producing plasma cells. **C.** Cellular immunity. Some of the lymphokines from the activated CD4 cells and the complex formed between antigens associated with class I MHC on altered self-cell and T-cytotoxic cell (CD8) receptors cause the activation of the CD8 cells, which mediate the cytotoxic killing of the altered self-cells. Some of the other lymphokines produced also play significant roles in hematopoiesis and activation of phagocytic cells. (Ag = antigen; APC = antigen-presenting cell; MHC = major histocompatibility complex; IL-1 = interleukin-1; IL-2 = interleukin-2; CSF = colony-stimulating factor; BFU-E = burst-forming unit-erythroid; GM-CFU = granulocyte/macrophage-colony-forming units; PMN = polymorphonuclear neutrophils; MAF = macrophage-activating factor.)

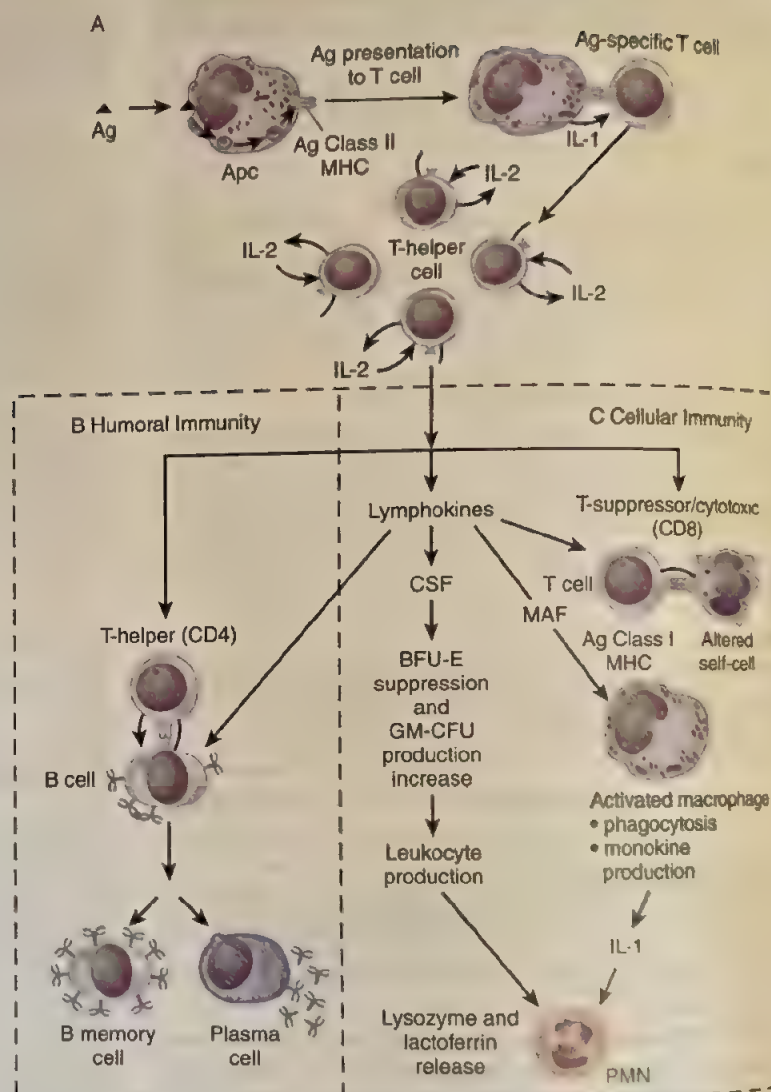


TABLE 7-7 Cytokines That Contribute to Inflammation

Cytokine	Target Cell	Activity
IL-1	T and B lymphocytes, macrophages, and tissue cells	Lymphocyte activation, macrophage activation, acute-phase reaction Decreases EPOR signaling
IL-2	T and B lymphocytes	Stimulates proliferation of T and mature B cells
IL-3 (multilineage colony-stimulating factor)	Stem cells, Basophils BFU-E	Stimulates differentiation of bone marrow stem cells Stimulates Basophil and BFU-E development (with EPO)
IL-4	B lymphocytes	B-cell proliferation
IL-5	B lymphocytes, eosinophils, precursor cells	B-cell growth and differentiation, eosinophil differentiation
IL-6	B lymphocytes, hepatocytes	Stimulates antibody production and acute-phase reactants Decreases EPOR signaling Inhibits iron release from macrophages
MIF	Macrophages	Inhibits migration

TABLE 7-7 Cytokines That Contribute to Inflammation—cont'd

Cytokine	Target Cell	Activity
MAF	Macrophages	Activates macrophages and enhances their functions
LCF	Phagocytes	Promotes chemotaxis to site of injury
LIF	Phagocytes	Inhibits migration
GM-CSF	Stem cells	Stimulates differentiation of granulocyte-monocyte precursor cells
M-CSF	Stem cells	Stimulates differentiation of monocytes
G-CSF	Stem cells	Stimulates differentiation of granulocytes
INF- γ	Macrophages	Activates macrophages for cytotoxic functions including erythrophagocytosis
		Induces MHC II molecules on APCs
		Increases myeloid cell production
		Downregulates EPOR
TNF- α	Macrophages, granulocytes	Activates macrophages, granulocytes, and cytotoxic cells
		Inhibits erythroid proliferation

IL = interleukin; MIF = macrophage migration inhibitory factor; MAF = macrophage activation factor; MHC = major histocompatibility complex; LCF = leukocyte chemotaxis factor; LIF = leukocyte inhibition factor; GM-CSF = granulocyte/macrophage-colony-stimulating factor; M-CSF = monocyte-colony-stimulating factor; G-CSF = granulocyte-colony-stimulating factor; APC = antigen-processing cell; INF- γ = interferon gamma; TNF- α = tumor necrosis factor-alpha.

Inflammation has an inhibitory effect on erythropoiesis by suppressing the efficacy of erythropoietin (EPO). This effect is twofold. Cytokines interfere with EPO-producing kidney cells and reduce the number and responsiveness of EPO receptors. The result is fewer red cells produced and increased apoptosis.

The final, and usually minor, factor in ACI is shortened red cell survival. Inflammation causes an increased expression of DMT1, stimulating uptake of iron into the macrophages. Higher levels of TNF- α and IL-6 have been demonstrated. This may cause damage to red cell membranes and stimulate erythrophagocytosis.²⁷

It has been suggested that the iron sequestration demonstrated in ACI is a part of the body's defense mechanism against pathogens.

ADVANCED CONTENT

Dysregulation of iron homeostasis, also called reticuloendothelial iron block, refers to the inability to use iron properly. It is a primary contributor to the pathogenesis of ACI (Box 7-5).^{39,40} Proinflammatory cytokines such as IL-1, IL-6, and TNF- α divert iron from the circulation into storage sites in the reticuloendothelial system.^{39,41} This effect of reducing the availability of iron for hematopoiesis produces a functional iron-deficiency state, despite having sufficient iron stores. A decrease in intestinal iron absorption and impaired iron reutilization by the hepatocytes also occurs in patients with ACI.⁴¹

BOX 7-5 Mechanisms Involved in Anemia of Chronic Inflammation

Dysregulation of Iron Homeostasis

- Inflammatory cytokines (IL-1, IL-6, and TNF- α) divert iron into storage sites
- Increased hepcidin decreases iron absorption and blocks iron release

Suppression of Erythropoiesis by Cytokines

- Cytokines (IL-1, IL-6, TNF- α and IFN- γ) increase apoptosis (cell death) in erythroid precursors

Blunted Erythropoietin Response

- Cytokines (IL-1, IL-6 and TNF- α) decrease erythropoietin release by kidneys

Decreased RBC Survival

- Extracorporeal defect
- Erythrophagocytosis by splenic and hepatic macrophages
- Damage from inflammation
- Coating with antibody and complement

Mechanical damage by fibrin

The dysregulation of iron homeostasis in ACI is mediated by hepcidin, an iron-regulating protein and acute phase reactant.^{27,41-43} Proinflammatory cytokines (IL-1, IL-6,

IL-10, IFN- γ , and TNF- α) increase production of hepcidin by the liver.⁴⁰ When inflammation or infection is present, hepcidin works to decrease the amount iron released from storage into circulation and increase iron retention intracellularly (increasing ferritin storage). Transferrin levels also decrease during inflammation to prevent the availability of nonheme iron.⁴¹ Hepcidin acts to decrease iron absorption from the small intestine and block iron release from macrophages.⁴⁴ Because iron is important for the growth of many bacteria, decreasing the availability of iron is likely a defense against infection, even though some bacteria might find it more accessible inside the macrophage itself.⁴¹ Ultimately, hepcidin plays a central role in the development of ACI by decreasing the availability of iron for hemoglobin synthesis.

Macrophages in the bone marrow demonstrate increased iron stores despite the low serum iron. Through hepcidin signaling, iron is trapped within the reticuloendothelial system and is unable to be fully utilized in erythropoiesis.⁴¹

Clinical Findings

Anemia may be present in as little as 30 days in critically ill individuals. ACI presents as a mild to moderate anemia. It has been found that as many as 70% of consecutive hospitalized elderly patients with anemia can be attributed to inflammation.³⁹

Laboratory Testing and Results

Anemia of chronic inflammation (ACI) is defined by an aggregate of clinical, morphological, and laboratory findings in Box 7-6. Differentiation between IDA and ACI is important and detailed in Table 7-8.

BOX 7-6 Indices and Features of Anemia of Chronic Inflammation

Clinical

- Anemia present for several months following development of a chronic disease

Morphological

- Usually microcytic or normocytic RBCs
- May be hypochromic or normochromic
- Normal number of bone marrow erythrocytic precursors
- Increased storage iron
- Decreased sideroblasts
- Rare to absent ringed sideroblasts

Laboratory

- Decreased serum iron
- Decreased total iron-binding capacity (TIBC)
- Decreased % transferrin saturation
- Normal to increased serum ferritin levels
- Normal serum soluble transferrin receptor levels

TABLE 7-8 Comparison of Anemia of Chronic Inflammation With Iron-Deficiency Anemia

Laboratory Finding	Normal	ACI	IDA
Hgb (g/dL)	Female – 12–16 Male – 14–18	↓	↓
MCV (fL)	80–100	N to ↓	↓
MCH (pg)	27–31	N to ↓	↓
MCHC (%)	32–36	N to ↓	↓
Reticulocytes (%)	0.5–2.0	↓	↓
Reticulocyte Hgb content	28–36 pg	N	↓
CRP	3–5 mg/L	↑	N
Serum iron (mcg/dL)	Female: 50–170 Male: 65–170	↓	↓
TIBC (mcg/dL)	250–450	↓	↑
Serum ferritin (ng/mL)	12–30	↑	↓
Transferrin saturation (%)	Female: 15–50 Male: 20–50	↓	↓
RE marrow iron deposits	2–3+	↑	↓
Sideroblasts (%)	40–60	↓	↓
sTfR	1.5–2.75 mg/L	↑	N to ↑
sTfR-ferritin index		<1	>2
FEP (mcg/dL)*	16–65	↑	↑
Hepcidin*	29–254 ng/mL Men 17–286 ng/mL Women	↑	N to ↓

Reference ranges as provided in the front cover of the text using the conventional units. ACI = anemia of chronic inflammation; IDA = iron-deficiency anemia; CRP = C-Reactive Protein; RE = reticulo-endothelial; sTfR = Soluble transferrin Receptor; FEP = free erythrocyte protoporphyrin; * not commonly performed

Peripheral Blood

On examination of the peripheral blood, the red blood cells may be microcytic to normocytic in size and hypochromic to normochromic in color (Fig. 7-11). Anisocytosis and poikilocytosis are slight to absent. The reticulocyte count does not reflect the degree of anemia. The WBC and platelet counts are variable, depending on the underlying condition.

Iron Studies

Patients with ACI have decreased serum iron and transferrin saturation while the ferritin levels are normal to increased. There is an increase in free erythrocyte protoporphyrin (FEP) or zinc protoporphyrin (ZPP), and unlike IDA, the TIBC is decreased because transferrin is a negative acute phase reactant. The differential diagnosis between iron deficiency anemia of inflammation can be made via measurement of

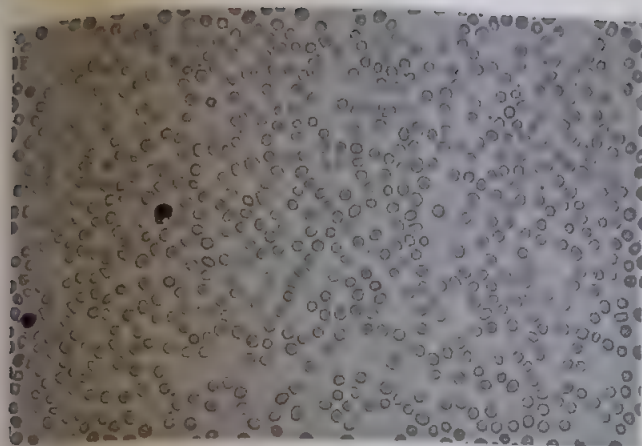


FIGURE 7-11 Normochromic, normocytic red blood cells in a patient with anemia of chronic inflammation (Wright's stain, $\times 200$).

plasma sTfR concentration. The sTfR is normal in patients with ACI because they have plenty of iron, though it is stuck in storage, while sTfR is elevated with IDA.^{27,38}

Bone Marrow

In the bone marrow, there is an adequate number of erythroid precursors, and the M:E ratio may be increased because of decreased erythropoiesis. Sideroblasts are decreased, but the macrophages have an increased amount of storage iron²⁷ (Fig. 7-12). In contrast, iron stores are decreased or absent in IDA.

Immune Response

Detection of inflammatory markers, such as C-reactive protein (CRP), can substantiate the presence of inflammation.²¹ In the future, measuring hepcidin, an acute phase reactant, may be useful in the detection of ACI.³¹

Treatment

When possible, treatment for ACI is to address the underlying disease first. In cases in which treating the underlying disease is not feasible, other options are necessary. The effect of treatment on the underlying disease must be considered. Conditions such as infection or cancer are of concern.



FIGURE 7-12 Increased reticuloendothelial iron in a patient with anemia of chronic inflammation (Prussian blue stain, $\times 200$).

Iron supplementation (orally or parenteral), erythropoiesis-stimulating agents, or a combination of both are commonly used therapies. For patients with ACI associated with chronic infection or malignancy, supplements of iron should be avoided. Erythropoiesis-stimulating agents for patients with ACI are currently approved for use in patients with renal failure or who are undergoing chemotherapy. The therapeutic effect stimulates erythropoiesis and suppresses hepcidin.

Transfusion is only considered in an emergency when the patient is clinically unstable and a rapid increase in hemoglobin is needed.²⁷

CRITICAL THINKING QUESTION

- 7-3** It is a given that red blood cells will be affected in anemia. What effect might be seen in white blood cells in anemia of chronic inflammation?

Sideroblastic Anemia

The **sideroblastic anemias** are a group of disorders characterized by abnormal iron utilization in heme synthesis resulting in the accumulation of iron in erythroid cells. This results in a hypochromic anemia, ineffective erythropoiesis, an increase in serum and tissue iron, and the presence of ringed sideroblasts in the bone marrow.

Etiology

It is a very diverse group of anemias and can be inherited or acquired. The inherited sideroblastic anemias include X-linked congenital sideroblastic anemia and autosomal recessive sideroblastic anemia. The acquired sideroblastic anemias can be primary or secondary (Box 7-7). Primary causes include myelodysplastic syndromes such as myelodysplastic syndrome with ringed sideroblasts (MDS-RS) (see Chapter 20). The secondary sideroblastic anemias are typically the result of toxins (such as lead), drugs (antibiotics such as chloramphenicol, hormones, or chemotherapy), copper deficiency, or chronic neoplastic disease.²⁶

Pathophysiology

Heme synthesis takes place in the mitochondria. The first step involves the condensation of glycine and succinyl-coenzyme

BOX 7-7 Common Causes of Sideroblastic Anemia

Inherited

- Congenital sideroblastic anemia, sex-linked
- Autosomal recessive sideroblastic anemia

Acquired

- Primary or idiopathic
- Myelodysplasia with ring sideroblasts (MDS-RS)
- Secondary
- Lead
- Alcohol
- Drugs, including isoniazid and chloramphenicol

A (succinyl-CoA) by aminolevulinic acid synthase (ALAS2) to form aminolevulinic acid (ALA) using a pyridoxal phosphate as a cofactor. The hereditary sideroblastic anemias are rare. The most common cause of congenital sideroblastic anemia has been identified as an X-linked mutation that results in a defect in ALAS2. While the congenital sex-linked type is more common, nearly 100 mutations have been identified to cause autosomal recessive sideroblastic anemia. A common subtype is in the SLC25A38 gene. The anemias typically appear in males within the first decades of life but may manifest in both males and females later in life.

The **ringed sideroblasts** are nucleated red blood cells in which iron is accumulated in the mitochondria that surround the nucleus (Fig. 7-13). These siderotic granules are visible with Prussian blue stain.^{26,45,46}

Lead poisoning due to lead paint and other exposures remains a common concern in the development of sideroblastic anemia, especially in children. Lead poisoning impairs enzymes involved in several steps of heme synthesis. The heme enzymes inhibited are D-aminolevulinate dehydrase and ferrochelatase. Coarse basophilic stippling of the RBCs is a common feature of lead poisoning due to precipitation of remnant ribosomes (Fig. 7-14).

Clinical Findings

Signs and symptoms of sideroblastic anemia can include weakness, fatigue, palpitations, shortness of breath, pale skin, and headaches. Hepatomegaly (enlarged liver) or splenomegaly (enlarged spleen) might also occur. In addition to sideroblastic anemia, cognitive impairment is also a grave concern with lead exposure.⁴⁷

Laboratory Testing and Results

The principal clinical feature of this anemia is the characteristic ringed sideroblasts in the bone marrow aspirates²⁶ (Box 7-8).

Peripheral Blood

The anemia is mild to severe. The red blood cells are dimorphic, ranging from microcytes to normocytes, and may appear hypochromic (Fig. 7-15). The MCV and MCH are often

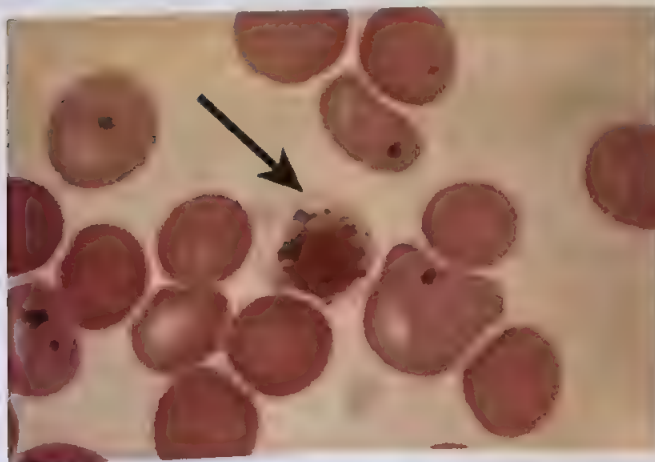


FIGURE 7-13 Ringed sideroblast as detected by Prussian blue staining of a bone marrow aspirate (Prussian blue stain, $\times 1,000$).

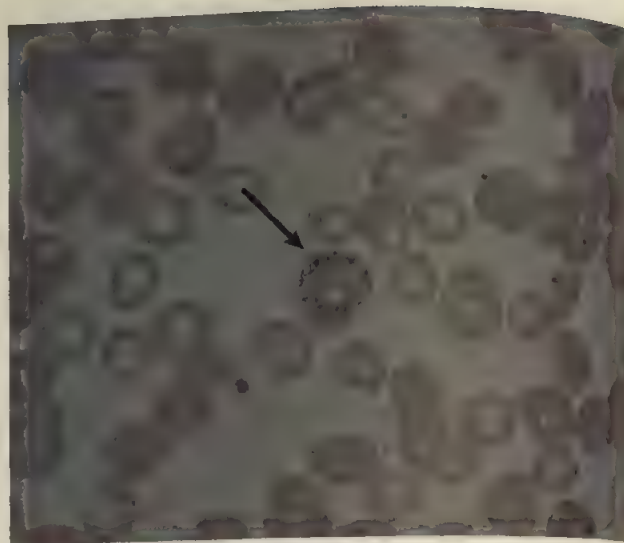


FIGURE 7-14 Prominent basophilic stippling is found with defective heme synthesis.

BOX 7-8 Clinical and Morphological Features of Myelodysplastic Syndrome With Ringed Sideroblasts (MDS-RS)*

Clinical

- Usually older than 50 years of age
- Weakness, pallor, fatigue associated with anemia

Morphological

- Dimorphic RBC population with prominent hypochromia
- Anisopoikilocytosis of RBCs, which may be associated with basophilic stippling
- Hypercellular bone marrow with erythroid hyperplasia
- More than 15% ringed sideroblasts

*Single Lineage (MDS-RS-SLD) or Multiple Lineage (MDS-RS-MLD) Dysplasia

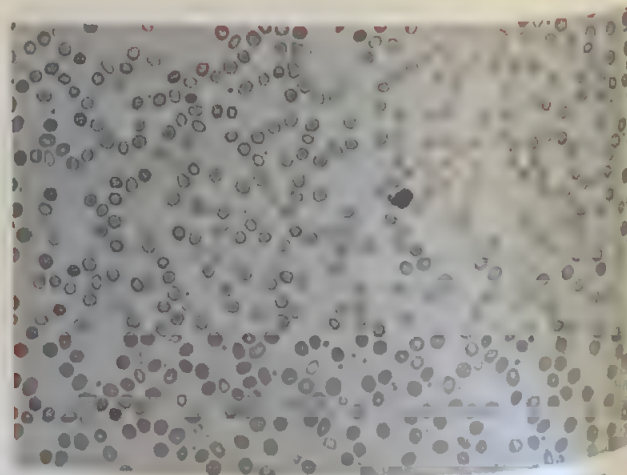


FIGURE 7-15 Dimorphic population of red blood cells with a striking hypochromic component in a patient with sideroblastic anemia. Moderate anisopoikilocytosis is also present (Wright's stain, $\times 200$).

decreased. The RDW is increased, representing the dual population of red blood cells. Occasionally, macrocytosis is present, and differentiation needs to be made from myelodysplastic syndromes that present with ringed sideroblasts. Other abnormalities of the red cells include anisocytosis, poikilocytosis, target cells, Pappenheimer bodies (iron deposits in the red cell), and basophilic stippling. The WBC and platelet counts are usually normal.

Iron Studies

Iron studies show increases in serum iron and increased ferritin, and an increase in percent transferrin saturation levels.⁴⁸ Serum soluble transferrin receptor levels are usually normal or decreased because there is plenty of iron present in the red cells, though it is not being incorporated into the protoporphyrin IX.⁴⁹

Bone Marrow

The primary feature common to all sideroblastic anemias is the presentation of the ringed sideroblast in the bone marrow. Ineffective erythropoiesis, erythroid hyperplasia, and increased stainable bone marrow iron are also characteristics of sideroblastic anemia.⁴⁶

In the myelodysplastic syndrome with ringed sideroblasts, the bone marrow is typically hypercellular with erythroid hyperplasia and dyserythropoiesis resulting in macrocytes (see Chapter 20). Iron stains reveal more than 15% ringed sideroblasts. Some patients may progress to a phase of marrow failure with some later displaying leukemic blasts.⁵⁰

Treatment

It is important to know whether the anemia is hereditary or acquired. In the case of secondary sideroblastic anemia

caused by drugs, rapid improvement can be seen with the discontinuation of the offending medication. Administration of pyridoxine (vitamin B₆) has also been proven to be effective in both acquired and inherited forms. If pyridoxine is not effective, transfusion is an option, but it can lead to transfusion related iron loading that may require iron chelation therapy.⁴⁸

The differential diagnosis comparing RBC indices and peripheral blood and bone marrow features of IDA, ACI, and sideroblastic anemia is presented in Table 7-9.

CRITICAL THINKING QUESTION

7-4 Basophilic stippling is a peripheral smear finding that can aid in the differentiation of sideroblastic anemia from the other microcytic/hypochromic anemias. Why is this only seen in sideroblastic anemia?

The Porphyrrias

The **porphyrias** are a group of rare inherited disorders that involve a block in porphyrin synthesis that is due to a defect in the enzymes in the pathway of heme synthesis. Note the precipitated porphyrins in the red blood cell and nucleated RBC cytoplasm in Fig. 7-16. This causes porphyrin heme precursors to accumulate in tissues, and large amounts are excreted in urine and feces. The porphyrias are classified as acute or nonacute, according to their clinical presentation, and as erythropoietic or hepatic, depending on the site of abnormal metabolism.

TABLE 7-9 Differential Diagnosis Comparing RBC Indices and Peripheral Blood and Bone Marrow Features of IDA, ACI, and Sideroblastic Anemia

Indices	Iron-Deficiency Anemia	Anemia of Chronic Inflammation	Sideroblastic Anemia
RBC count	Decreased	Decreased	Decreased
Hemoglobin (Hgb) concentration	Decreased	Decreased	Decreased
Hematocrit (Hct)	Decreased	Decreased or normal	Variable
MCV	Decreased	Normal or decreased	Variable
MCH	Decreased	Usually normal or may be decreased	Variable
MCHC	Decreased	Usually normal or may be decreased	Variable
Red cell distribution width (RDW)	Increased	Usually normal or may be increased	Normal or increased
Peripheral Blood and Bone Marrow Features			
Anisocytosis	Yes	No	Yes*
Poikilocytosis, including target cells	Yes	No	Yes*
Basophilic stippling	No	No	Yes
Stainable bone marrow iron	Decreased or absent	Increased	Increased
Marrow sideroblasts/ringed sideroblasts	Decreased	Decreased	Increased

*In hereditary forms.

MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration.

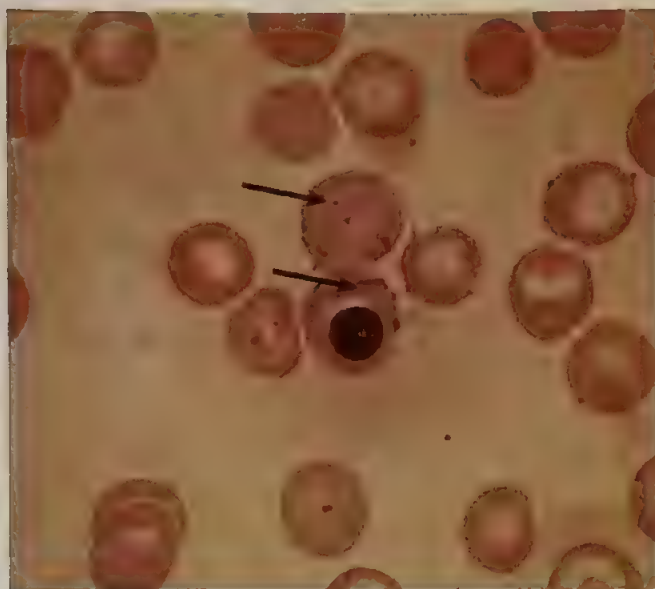


FIGURE 7-16 Erythropoietic porphyria. Note the precipitated porphyrins in the cytoplasm. (From Bell A. Hematology. In Listen, Look and Learn. Bethesda, MD: Health and Education Resources, Inc., with permission.)

The porphyrias are associated with neurovisceral attacks, which include photosensitivity, motor dysfunction, sensory loss, mental disturbances, and sometimes abdominal pain. The classification and characteristics of the porphyrias are summarized in Table 7-10. The most common is acute intermittent porphyria characterized by colicky abdominal pain, vomiting, diarrhea, constipation, and central nervous system involvement.⁵¹

Iron Overload and Hemochromatosis

Iron overload is defined as the accumulation of excess iron in reticuloendothelial cells in various tissues. **Hemochromatosis**

describes a clinical disorder that results in tissue damage resulting from excess iron. In this disorder of iron storage, there is an inappropriate increase in intestinal iron absorption that leads to excess iron in the tissues.

Etiology

Iron overload may be primary (inherited) or secondary to the chronic anemias and their treatment. The excess iron is stored in the liver, heart, and pancreas, and damages these organs.⁵² The different types of hemochromatosis are summarized in Box 7-9.

Hereditary Hemochromatosis

Hereditary hemochromatosis (HH) is a recessive genetic disorder and is one of the most frequent genetic diseases in North America, typically found in those with Northern European ancestry, affecting approximately 1 in 300 people. Prevalence is especially high in Ireland, United Kingdom, France, and Denmark.^{53,54}

Secondary Hemochromatosis

Secondary hemochromatosis can be acquired or secondary to other inherited hemolytic anemias. The common characteristics of secondary hemochromatosis are anemia, ineffective erythropoiesis, and iron overload. Signaling molecules associated with these disorders decrease hepcidin regulation of ferroportin.⁵² Secondary hemochromatosis in these patients is also due to the fact that they have received repeated transfusions. This leads to increased iron storage because there is no mechanism for iron excretion. Phlebotomy is not appropriate for individuals exhibiting ineffective erythropoiesis. Iron overload from transfusion therapy is treated with chelation therapy.⁵⁵

African Iron Overload

A distinct iron-loading disorder is prevalent in persons of sub-Saharan African descent, affecting up to 10% of some rural populations. These individuals have a predisposition to iron overload as a result of excessive dietary iron intake.

TABLE 7-10 Classification and Characteristics of the Porphyrrias

Disease	Deficient Enzyme	Course	Inheritance	Erythroid/Hepatic	Symptoms
Acute intermittent porphyria	Porphobilinogen deaminase	Acute	Autosomal dominant	H	Neurological
Hereditary coproporphyria	Coproporphyrinogen III oxidase	Acute	Autosomal dominant	H	Neurological Photosensitive
Variegate porphyria	Porphobilinogen oxidase	Acute	Autosomal dominant	H	Neurological Photosensitive
Cutaneous Hepatic porphyria	Uroporphyrinogen decarboxylase	Chronic	Variable	H	Photosensitive
ALA dehydratase deficiency porphyria	ALA dehydratase	Acute	Autosomal recessive	E	Neurological
Congenital erythropoietic porphyria	Uroporphyrinogen III cosynthetase	Chronic	Autosomal recessive	E	Photosensitive
Erythropoietic protoporphyria	Heme synthetase	Chronic	Autosomal dominant	E	Photosensitive

ALA = aminolevulinic acid; E = erythroid; H = hepatic.

BOX 7-9 Classification of Hemochromatosis**Hereditary Hemochromatosis**

- Classical hemochromatosis type 1
- Juvenile type 2
- Transferrin receptor type 3
- African overload type 4

Secondary Hemochromatosis

- Hereditary disorders
- Thalassemia
- Sickle cell anemia
- Sideroblastic anemia
- Enzyme-deficiency anemia
- Hereditary spherocytosis

Acquired Disorders

- Anemia not due to blood loss in which multiple transfusions are required
- Dyserythropoietic anemia

particularly related to a traditional African beer containing iron leached from the drums used in brewing. However, not everyone develops iron overload.⁵⁶ African iron overload is not due to mutations of the *HFE* gene but is associated with mutations in the *SLC40A1* gene. This gene is responsible for the production of ferroportin. It is thought that these mutations may account for the iron overload found in some individuals.⁵⁷

A summary and comparison of iron studies in the microcytic hypochromic anemias and hemochromatosis are provided in Table 7-11.

Pathophysiology

The disease is caused by excessive absorption of iron due to a hepcidin deficiency and a gradual accumulation of iron in the tissues, which leads to chronic liver disease, arthritis, diabetes, pituitary damage, congestive cardiac failure, and cardiac arrhythmias (Fig. 7-17). Hyperpigmentation, described as “bronze skin,” may be an early symptom as melanin production in the skin is stimulated by iron deposits. Patients with HH absorb two to three times as much dietary iron as normal individuals. Most cases of hereditary hemochromatosis involve ineffective or diminished hepcidin regulation

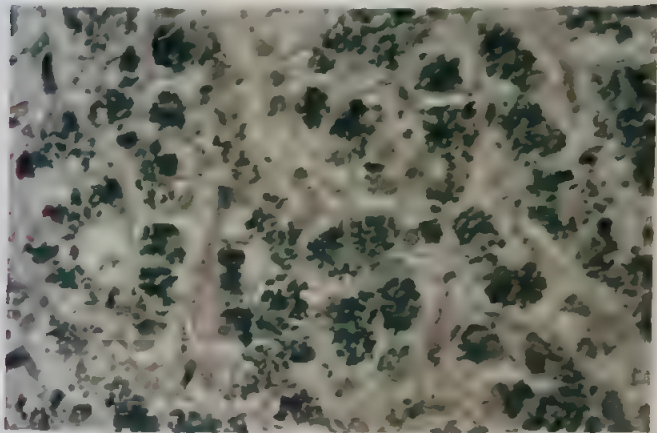


FIGURE 7-17 Liver biopsy from a patient with idiopathic hemochromatosis and cirrhosis. Note the excess deposits of iron (Ferric ferricyanide stain).

of ferroportin, resulting in excessive absorption of iron with adequate iron stores.⁵⁸

ADVANCED CONTENT

There are four types of hereditary hemochromatosis. Type 1 is the most common and caused by mutations in the *HFE* gene. More than 100 mutations in the *HFE* gene have been shown to cause type 1 hemochromatosis. One such mutation is a single-base change that results in the substitution of tyrosine for cysteine at position 282 of the *HFE* protein (C282Y). The C282Y mutation alters the conformation of the *HFE* protein and interferes with its function of regulation of iron absorption.^{17,49,53} Two other *HFE* mutations, H63D and S65C, have been described in combination with C282Y and can be tested for. Neither alone typically has a pathological effect in the heterozygous or homozygous state. There are other mutations not associated with the *HFE* gene. Type 2 is caused by mutations of the hemojuvelin gene (*HJV*) or hepatic antimicrobial protein gene (*HAMP*). Type 3 is caused by mutations in the transferrin receptor 2 gene (*TfR2*). Types 1 to 3 result in a hepcidin deficiency. Type 4 is caused by a mutation in the ferroportin 1 gene (*FPN1*) that results in resistance to the action of hepcidin.⁵³

TABLE 7-11 Summary and Comparison of Iron Studies in Microcytic-Hypochromic Anemias and Hemochromatosis

Disorder	Iron	Serum Ferritin	Serum TIBC	Transferrin Saturation	Transferrin Receptor	ZPP	Stainable Bone Marrow Iron
Iron deficiency anemia	D	D	I	D	I	I	Absent
Anemia of chronic inflammation	D	I	N/D	N/D	N/D	I	N/I
Sideroblastic anemia	I	I	N/D	I	N/D	V	I
							Ringed sideroblast
Hereditary hemochromatosis	I	I	N/D	I	N/D	N	I

D = decrease, I = increase, N = normal, V = variable TIBC = total iron binding capacity, ZPP = zinc protoporphyrin

Clinical Findings

Signs and symptoms of HH usually occur in midlife. The most common complaint is joint pain. Chronic arthritis of the second and third metacarpophalangeal joints is highly suggestive of the disease. Early symptoms are usually nonspecific. They may include fatigue, arthralgia, bronze discoloration of the skin, and erectile dysfunction. As the disease progresses, hepatomegaly develops, which leads to cirrhosis and fibrosis of the liver. Iron deposit in the heart tissue causes cardiomyopathy. Other complications are diabetes mellitus, hypopituitarism, hypogonadism, and hypoparathyroidism.^{49,53}

Laboratory Testing and Results

Although iron metabolism is abnormal, erythropoiesis is normal, and hematologic abnormalities are usually not seen. Other laboratory abnormalities include increased liver function enzyme tests, particularly alanine and aspartate aminotransaminases (ALT, AST).

In patients with HH, serum iron, serum ferritin, and serum transferrin levels are increased. Serum ferritin levels

>200 ng/mL and transferrin saturation >45% in females and ferritin >300 ng/mL and transferrin saturation >50% in males suggest HH. Diagnosis can be confirmed by direct analysis of the common HFE gene mutations.⁴⁷

Treatment

The goal of treatment of iron storage disease is to remove the excess amount of iron from the body. Patients who have symptoms of HH require therapeutic phlebotomy. Initial removal of 500 mL of blood once or twice a week is performed until there is a marked decrease in serum transferrin percent saturation and serum ferritin. The goal is to have the serum ferritin less than 50 ng/mL. Once this is achieved, phlebotomy frequency is reduced to two to four times per year. Patients with severe anemia or congestive heart failure may not tolerate phlebotomy. In the event that therapeutic phlebotomy is not appropriate, one of three FDA-approved chelating agents may be used to reduce iron stores.^{49,53}

SUMMARY CHART

- The majority of the total body iron is present as hemoglobin iron affecting RBC capacity to carry oxygen.
- Ferrous iron (Fe^{2+}) is easily absorbed into the enterocytes (intestinal mucosa cells) via Divalent Metal Transporter 1 (DMT1).
- Ferritin is the primary storage compound for iron. Hemosiderin is another storage form of iron.
- Transferrin is a globulin protein responsible for transporting iron within the bloodstream.
- Ferrous iron combines with protoporphyrin IX via ferrochelatase in the mitochondria of the red blood cell to form heme.
- Ninety percent of tissue iron is present as storage iron in the form of ferritin or hemosiderin.
- Serum ferritin levels are an indirect measure of iron stores in the body.
- The transferrin receptor is a tool for the assessment of iron. The sTfR level is inversely proportional to the amount of iron in the body.
- Iron deficiency occurs when there is inadequate iron intake, excess iron loss, or increased need for iron.
- Increased iron consumption states include growth spurts, menstruation, pregnancy, lactation, and iron deficiency.
- Iron deficiency anemia (IDA) presents as a hypochromic and microcytic anemia with an increased RDW.
- Iron studies for patients with IDA include a decreased serum iron, serum ferritin and percent transferrin saturation, and increased TIBC and increased transferrin receptor.
- Anemia of chronic inflammation (ACI) results from iron being trapped in the macrophages resulting from increased acute phase reactant hepcidin, hemolysis, and ineffective levels of EPO.
- Iron studies of patients with anemia of chronic inflammation include decreased iron, normal to decreased iron binding, decreased percent transferrin saturation, increased ferritin, and a normal TfR level.
- Bone marrow evaluation of anemia of chronic inflammation reveals increased storage iron and decreased sideroblastic iron.
- Sideroblastic anemias are disorders of heme synthesis and may be inherited or acquired.
- Lead poisoning impairs enzymes D-aminolevulinic acid dehydratase and ferrochelatase involved in heme synthesis.
- Sideroblastic anemia presents with the "ringed sideroblast" due to iron being blocked from incorporation to form heme. It can be demonstrated with a Prussian blue stain.
- Iron studies of patients with sideroblastic anemia include increased iron, normal to decreased iron binding, increased percent transferrin saturation, increased ferritin, and a normal TfR level.
- Porphyrias are a group of rare (usually hereditary) diseases that result in errors in heme synthesis.
- Hereditary hemochromatosis is an inherited iron overload disorder.
- In hereditary hemochromatosis, serum iron, serum ferritin, and serum transferrin saturation levels are all increased.
- Secondary hemochromatosis can be acquired or secondary to other hereditary hemolytic anemias. It is most commonly caused by repeated transfusions.
- The most common HFE gene mutation found in hereditary hemochromatosis is C282Y.

CASE STUDY 7-1

A 76-year-old male presented to his physician feeling exceedingly tired with a loss of energy. He has a history of arthritis that has been treated with nonsteroidal anti-inflammatory drugs (NSAIDs). He became concerned when he started having abdominal cramps and noted black stools. On physical exam he appeared pale with a rapid pulse. The CBC results were as follows:

WBC	$10.8 \times 10^9/L$
RBC	$3.02 \times 10^{12}/L$
HGB	6.8 g/dL
HCT	22.1%
MCV	73.2 fL
MCH	22.5 pg
MCHC	30.8 g/dL
RDW	20.2%
PLT	$172 \times 10^9/L$

Peripheral smear showed moderate anisocytosis, microcytes, and hypochromasia.

QUESTIONS

- Which of the following disorders is LEAST likely to cause this anemia?
 - Hereditary hemochromatosis
 - Lead poisoning
 - Iron deficiency anemia
 - Sideroblastic anemia

- What additional tests may be useful in confirming the diagnosis?
 - Measurement of serum iron, ferritin, TIBC, and transferrin saturation
 - Hemoglobin electrophoresis
 - Reticulocyte count
 - Indirect bilirubin
- With the following results, what is the most likely cause of this anemia?
 - Serum Iron – Low
 - TIBC – High
 - % Transferrin Saturation – Low
 - Ferritin – Low
 - Anemia of chronic inflammation due to arthritis
 - Sideroblastic anemia
 - Hemolytic anemia
 - Iron deficiency anemia due to GI bleed

ANSWERS

- a
- a
- d

CASE STUDY 7-2

A 3-year-old girl presented in the emergency department with a 2-day history of abdominal pain and constipation. Patient had no significant past medical history. Her general physical examination was unremarkable. CBC results were as follows:

WBC	$14.5 \times 10^9/L$
RBC	$4.01 \times 10^{12}/L$
HGB	9.7 g/dL
HCT	30.9%
MCV	77.1 fL
MCH	24.2 pg
MCHC	31.4 g/dL
RDW	15.5%
PLT	$279 \times 10^9/L$

Peripheral smear showed a dimorphic population with microcytic/hypochromic cells, moderate coarse basophilic stippling, and a rare nucleated RBC.

QUESTIONS

- What clinical disorder do you suspect?
 - Iron deficiency anemia
 - Sideroblastic anemia

- Anemia of chronic inflammation
- Thalassemia
- If serum iron studies were performed on this patient, they would reveal:
 - Decreased serum iron, decreased ferritin, and decreased percent transferrin saturation
 - Decreased serum iron, increased ferritin, and decreased percent transferrin saturation
 - Increased serum iron, increased ferritin, and increased percent transferrin saturation
 - Increased serum iron, normal ferritin, and normal percent transferrin saturation
- What other laboratory test may be helpful in confirming the cause of this anemia?
 - Copper level
 - EPO activity
 - Lead level
 - Vitamin B₁₂ level

ANSWERS

- b
- c
- c

CASE STUDY 7-3

A 56-year-old diabetic man visited his physician because he was experiencing fatigue, joint stiffness, and noticed increased pigmentation on his arms and legs. On physical exam it was noted that he has a cardiac arrhythmia and hepatomegaly.

Laboratory results of the CBC are as follows:

WBC	$5.9 \times 10^9/L$
RBC	$4.97 \times 10^{12}/L$
HGB	15.2 g/dL
HCT	46.2%
MCV	93.0 fL
MCH	30.6 pg
MCHC	32.9 g/dL
RDW	15.7%
PLT	$336 \times 10^9/L$

Liver enzymes were elevated.

QUESTIONS

- What is the most likely cause of this patient's disorder?
 - Iron deficiency anemia
 - Anemia of chronic inflammation
 - Acquired sideroblastic anemia
 - Hereditary hemochromatosis
- Serum iron studies would be helpful to diagnosis this patient. What do you expect the serum iron studies in this patient to reveal?
 - Increased serum iron, increased serum ferritin, and increased percent transferrin saturation
 - Decreased serum iron, normal serum ferritin, and decreased percent transferrin saturation
 - Decreased serum iron, decreased serum ferritin, and decreased percent transferrin saturation
 - Increased serum iron, decreased serum ferritin, and increased percent transferrin saturation

- There have been numerous genetic mutations described to cause hereditary hemochromatosis. What is the most common mutation in hereditary hemochromatosis?
 - C282Y
 - SLC40A1
 - TfR1
 - FPN1
- What is the appropriate therapy or treatment for this disorder?
 - Blood transfusion
 - Chelation therapy
 - Phlebotomy
 - Iron supplements

ANSWERS

- d
- a
- a
- c

CASE STUDY 7-4

A 42-year-old woman presented to her physician with pain in both of her wrists and in the proximal joints of both of her hands. She had also recently begun experiencing right knee pain. Her laboratory results revealed the following:

WBC: $10.0 \times 10^9/L$
 Hgb: 8.5 g/dL
 Hct: 25%
 MCV: 90 fL
 RDW: 12.5% (normal 11%–14%)
 Erythrocyte sedimentation rate (ESR): 100 mm/h (normal 0–10 mm/h)
 Rheumatoid factor: Positive

QUESTIONS

- What is the patient's diagnosis?
- What could cause the increase in ESR?
- Why was the patient anemic?

ANSWERS

- Anemia of chronic inflammation
- The increased inflammation associated with a diagnosis of RA
- Due to the acute phase reactants, mainly Hepcidin, working to restrict the body from absorbing and utilizing iron

REVIEW QUESTIONS

- Two-thirds of the total body iron is present as:
 - Hemosiderin
 - Ferritin
 - Transferrin
 - Hemoglobin iron
- Dietary iron is absorbed predominantly in the:
 - Stomach
 - Duodenum and first part of the jejunum
 - Transverse colon
 - Sigmoid colon
- The protein responsible for transporting iron in the bloodstream is:
 - Hemoglobin
 - Hemosiderin
 - Transferrin
 - Ferritin
- The hypochromic anemias represent a related group of disorders with:
 - A quantitative defect in hemoglobin synthesis
 - A qualitative defect in globin protein chains
 - Excess hemoglobin synthesis
 - Vitamin B₁₂ and folate deficiency
- The most common cause of hypochromic anemia is:
 - Sideroblastic anemia
 - Megaloblastic anemia
 - Iron deficiency anemia
 - Lead poisoning
- Microcytosis of red blood cells is reflected by:
 - Increased MCV
 - Decreased MCV
 - Increased hemoglobin concentration
 - Decreased hemoglobin concentration
- In anemia of chronic inflammation, hepcidin levels:
 - Increase, activating transferrin
 - Increase, inhibiting ferroportin
 - Decrease, inhibiting erythroferrone
 - Decrease, activating ferroportin
- A common feature of sideroblastic anemia is which of the following?
 - Ringed sideroblasts
 - Decreased iron
 - Decreased ferritin
 - Macrocytes
- Why are zinc protoporphyrin (ZPP) levels elevated in IDA, ACI, and sideroblastic anemias?
 - Hepcidin is a negative acute phase reactant
 - Iron is not incorporated into protoporphyrin IX
 - Hemochromatosis results in an accumulation of iron
 - Lack of intrinsic factor impairs iron absorption
- Prussian blue staining identifies:
 - Basophilic stippling
 - Heinz bodies
 - Siderotic granules
 - Reticulocytes
- Lead poisoning
 - Inhibits absorption of dietary iron
 - Inhibits nuclear maturation of red blood cells
 - Activates hepcidin production
 - Inhibits the ability of ferrochelatase to catalyze the insertion of iron into the protoporphyrin IX
- A disorder that results from the deficiency of an enzyme needed for protoporphyrin IX production is:
 - Porphyria
 - Hemochromatosis
 - Iron deficiency
 - Aplastic anemia
- The most common cause of secondary hemochromatosis is:
 - Drugs
 - Diet
 - Repeated blood transfusions
 - Malabsorption

See answers at the back of this book.

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Megaloblastic Anemias and Other Macrocytic Anemias

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CHAPTER OUTLINE

Etiology: Biochemical Aspects

Clinical Manifestations

Hematologic Features

- Ineffective Hematopoiesis
- Bone Marrow Morphology
- Peripheral Blood Morphology

Etiology: B₁₂ and Folic Acid Deficiency

- Vitamin B₁₂ Deficiency
- Folic Acid Deficiency

Laboratory Diagnosis of Megaloblastic Anemia

- Laboratory Tests for the Diagnosis of Vitamin B₁₂ and Folic Acid Deficiencies

Treatment

- Therapy for Vitamin B₁₂ Deficiency
- Therapy for Folic Acid Deficiency
- Response to Therapy

Macrocytic Nonmegaloblastic Anemias

Vitamin-Independent Megaloblastic Changes

- Inherited
- Acquired
- Drug and Toxin Induced

Summary Chart

Case Study 8-1

Case Study 8-2

Case Study 8-3

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 8-1 Define megaloblastic anemia.
- 8-2 List the common clinical signs and symptoms of megaloblastic anemia.
- 8-3 Compare and contrast the morphological characteristics of megaloblasts and normoblasts.
- 8-4 Describe the bone marrow morphology of megaloblastic anemia.
- 8-5 Distinguish the peripheral blood morphology of megaloblastic anemia from normal morphology.
- 8-6 List key facts about the absorption and metabolism of vitamin B₁₂ and folic acid.
- 8-7 Outline the causes of vitamin B₁₂ and folic acid deficiencies.
- 8-8 Compare and contrast pernicious anemia with the other types of vitamin B₁₂ deficiency, including their pathophysiology and clinical and laboratory findings.
- 8-9 Determine which individuals are at risk for megaloblastic anemia.
- 8-10 Given laboratory tests results for assessing megaloblastic anemia, predict a differential diagnosis of megaloblastic anemia.
- 8-11 Compare treatment options for patients with vitamin B₁₂ deficiency and those with folic acid deficiency.
- 8-12 List other causes of macrocytic nonmegaloblastic anemias.

This chapter discusses megaloblastic anemias that share some common morphological characteristics. The biochemical aspects of megaloblastic anemia along with clinical symptoms, laboratory findings, and treatment options are presented. Also covered are nonmegaloblastic anemias that exhibit similarities and are often confused with megaloblastic anemia upon diagnosis.

Etiology: Biochemical Aspects

Megaloblastic anemia is a subgroup of macrocytic anemia characterized by defective nuclear maturation caused

by impaired deoxyribonucleic acid (DNA) synthesis. The impairment of DNA synthesis results in an imbalance of maturation between the nucleus and cellular cytoplasm of rapidly dividing hematopoietic precursors. This results in the formation of abnormally large red blood cell precursors (megaloblasts) with maturation arrest and early destruction or apoptosis.^{1,2} This defect is manifested by the presence of megaloblasts in the bone marrow and macroovalocytes in the peripheral blood. The granulocyte precursors tend to be larger than normal, with giant metamyelocytes as a striking feature. An abnormal nuclear pattern in megakaryocytes may be seen

in severe anemia. The megaloblastic changes are not limited to the hematopoietic cells; changes are also present in other nucleated actively proliferating cells, such as skin, vaginal, uterine, cervical, and buccal cells.

All conditions that give rise to megaloblastic changes are caused by a decrease of the availability of the four immediate precursors of DNA.³ There is marked reduction in intracellular 5,10-methylene tetrahydrofolate, which is required to transform deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). This reaction is mediated by thymidylate synthase and is essential to maintain the normal rate of DNA synthesis (Fig. 8-1).⁴

The primary causes of lack of thymidine and consequently defective DNA synthesis are vitamin B₁₂ and folic acid deficiencies. These vitamins, in the form of cofactors, play important roles in some key reactions involved in DNA synthesis. In addition, drugs that interfere with the metabolism of these vitamins also cause DNA impairment. This impaired synthesis causes fragmentation of the nucleus and, ultimately, destruction of immature cells.

Clinical Manifestations

Certain clinical manifestations are common to all patients with megaloblastic anemias regardless of the cause. The degree of anemia may be mild to severe, with the symptoms of weakness, fatigue, shortness of breath, and lightheadedness. Congestive heart failure may or may not be present, depending on the degree of anemia. In severe anemia, the patient may have a lemon-yellow skin tint because of mild jaundice and pallor. Increased bilirubin is reported in patients as a result of intramedullary hemolysis caused by ineffective erythropoiesis.^{2,6}

Hematologic Features

Aspects of the hematologic features of megaloblastic anemia include ineffective hematopoiesis, bone marrow morphology, and peripheral blood morphology.

Ineffective Hematopoiesis

Megaloblastic anemia is associated with ineffective erythropoiesis and hemolysis. The mean corpuscular volume (MCV) is greater than 100 femtoliters (fL). Patients with megaloblastic anemia may have MCV values as high as 160 fL. This elevated MCV reflects the megaloblastic picture of the bone marrow. Increased erythrocyte precursors in the bone marrow and decreased red cell release into the peripheral blood are indicative of ineffective erythropoiesis, which is supported by decreased reticulocytes.

Megaloblastic erythrocyte progenitors have a much shorter life span than normal erythrocyte progenitors. They are more fragile and, therefore, die prematurely in the marrow. Evidence of intramedullary hemolysis includes decreased haptoglobin, increased levels of serum bilirubin, serum lactate dehydrogenase (LD, in particular, LD-1 and LD-2 isomers), and increased serum iron.^{5,7} Cell death occurs primarily at the later stages of the megaloblast maturation (i.e., basophilic and polychromatophilic stages), causing a decrease in production and release of mature erythrocytes. A decreased level of erythrocytes in the circulation stimulates EPO release, which in turn stimulates production of red cell progenitors.⁸

Ineffective granulopoiesis is defined by increased bone marrow white blood cell precursors and failure to release mature forms into the peripheral blood. The giant metamyelocytes do not mature to circulating neutrophils but, rather, die prematurely in the bone marrow. Ineffective thrombopoiesis is manifested by the presence of increased abnormal megakaryocytes in the bone marrow and thrombocytopenia in the peripheral blood.

Bone Marrow Morphology

Patients with megaloblastic anemia have a hypercellular bone marrow. The myeloid-to-erythroid (M:E) ratio is decreased (Fig. 8-2) and may be as low as 1:1 to 1:3. The degree of increased cellularity (megaloblastic picture) depends on the severity of the anemia. Megaloblasts are large cells with increased RNA per DNA unit. Their nuclear chromatin appears loose and less mature than the nuclear chromatin of the normal red cells at the same stage of maturation (Fig. 8-3). The cytoplasm maturation is, however, normal. This phenomenon is referred to as nuclear to cytoplasm **asynchrony**. The mature megaloblastic red cells entering the circulation usually have a shorter life span than normal, mature red cells.

Megaloblastic changes are manifested in white cell precursors by the presence of large bands (see Fig. 8-2B) and giant metamyelocytes in the bone marrow. The nucleus of the giant bands may show abnormal staining characteristics. These white cell abnormalities can sometimes be misdiagnosed for myelodysplastic syndrome due to nuclear asynchrony or sometimes even acute leukemia due to increased proerythroblasts.⁹ Megakaryocytes are also affected in severe megaloblastic anemia. They may have abnormal nuclear or cytoplasm morphology, such as increased nuclear lobulation and hypogranulation.¹⁰

Peripheral Blood Morphology

Megaloblastic anemia is a macrocytic, normochromic anemia. Depending on the degree of anemia, the MCV may range from 100 to 160 fL. The mean corpuscular hemoglobin (MCH) is

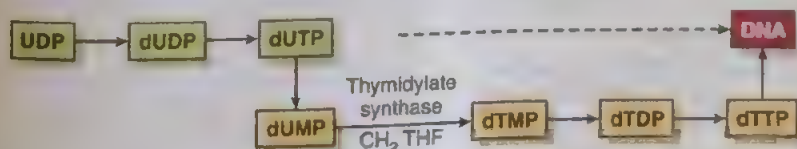


FIGURE 8-1 Thymidine synthesis pathway from uridine nucleotide. Uracil is incorporated into DNA in the absence of thymine. UDP - uridine diphosphate; dUDP - deoxyuridine diphosphate; dUTP - deoxyuridine triphosphate; dUMP - deoxyuridine monophosphate; dTMP - deoxythymidine monophosphate; dTDP - deoxythymidine diphosphate; dTTP - deoxythymidine triphosphate; CH₂ THF - methylene tetrahydrofolate.

vitamin B₁₂ is low and the storage rate is high, it takes several years for a person to develop vitamin B₁₂ deficiency because of malabsorption.

ADVANCED CONTENT

Structure

Vitamin B₁₂ (cobalamin) is a large, water-soluble molecule. It consists of a corrin nucleus composed of four pyrrole rings (A to D) with a cobalt atom at the center (Fig. 8-8), similar to the porphyrin structure. The corrin ring is attached to the nucleotide 5,6-dimethylbenzimidazole. The cobalt atom can be attached to several different molecules, such as adenosyl (5-deoxyadenoside), methyl, cyanide, and hydroxy, to form the biologically active forms of cobalamins (see Fig. 8-8).

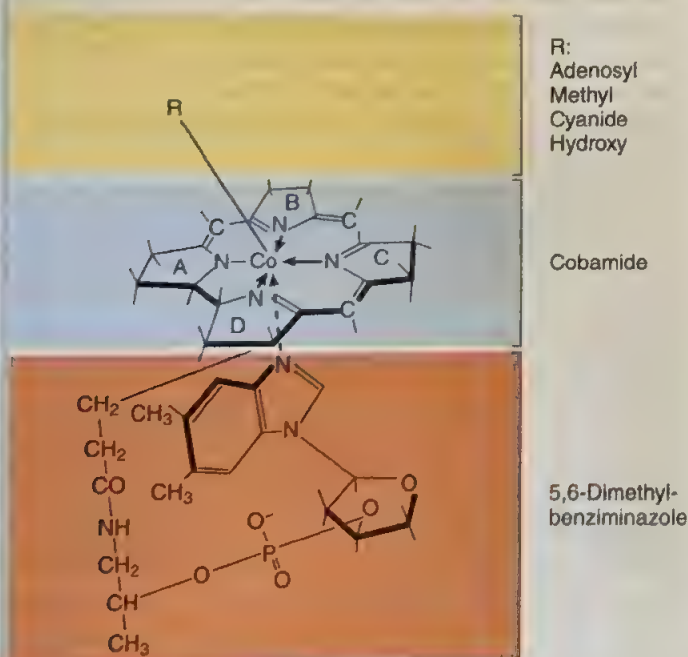


FIGURE 8-8 Structure of vitamin B₁₂ (cobalamin). When R is adenosyl, the compound is adenosylcobalamin (AdoCb); when R is methyl, it is methylcobalamin (MeCb); when R is cyanide, it is cyanocobalamin (CNCb); and when R is hydroxy, it is hydroxycobalamin (OHCb).

Transport and Metabolism

Vitamin B₁₂ is released from food and bound to **haptocorrin (HC)** from salivary glands to begin the process of active absorption. As this occurs, gastric parietal cells produce hydrochloric acid and **intrinsic factor (IF)** to prepare the stomach and intestine for absorbing this important vitamin. In the stomach, the salivary HC protects the B₁₂ molecule from acid degradation, but once it makes it to the duodenum, the protein is partially degraded by pancreatic trypsin to prepare for transfer to IF, which is even more resistant to proteolysis.¹³

From here, two important proteins are involved in the transport of vitamin B₁₂ from the duodenum to the ileum and from the ileum to tissues: the intrinsic factor (IF) and **transcobalamin**. IF is secreted by parietal cells of the stomach. IF

binds to vitamin B₁₂ and forms B₁₂-IF complex, which allows vitamin B₁₂ to be absorbed through the receptors present on the brush borders of the ileum (the distal half of the small intestine) (Fig. 8-9). The B₁₂-IF complex is taken into ileal enterocytes, where B₁₂ is released while the IF is destroyed. IF is essential for the absorption of B₁₂ taken orally at physiological dosages.⁵

ADVANCED CONTENT

Three carrier proteins provide the mechanisms for absorption, transport, and cellular uptake of vitamin B₁₂: **haptocorrin** (formerly transcobalamin I), **gastric intrinsic factor (IF)**, and **transcobalamin** (formerly transcobalamin II).¹⁴ The B₁₂-IF receptor in the ileum progressively increases until the maximum number of receptors ends near the terminal ileum. When vitamin B₁₂ leaves the ileum and enters the portal vein for plasma transport, it attaches to **haptocorrin (HC)** and **transcobalamin (TC)**. Ninety percent of newly absorbed B₁₂ is bound to TC, which rapidly delivers the vitamin to the liver, hematopoietic cells, and other dividing cells (see Fig. 8-9). Some B₁₂ binds to HC, which has the greatest affinity for B₁₂; this prevents its loss from the plasma. B₁₂ bound to HC is a passive reservoir in equilibrium with body stores in the liver but not taken up by other cells of the body. After initial absorption, 70% to 80% of B₁₂ is bound to HC while 10% to 30% is bound to TC.¹⁴ TC is synthesized by ileal cells, endothelial cells, liver, spleen, heart, and macrophages, and is secreted into the plasma.⁵ TC-bound B₁₂, also known as **holotranscobalamin (holoTC)**, is the biologically available fraction, whereas HC-bound B₁₂ is not available to cells outside the liver.¹⁴ Deficiency of TC causes megaloblastic anemia; yet lack of HC is not accompanied by anemia or megaloblastosis but shows decrease of B₁₂ levels.⁴

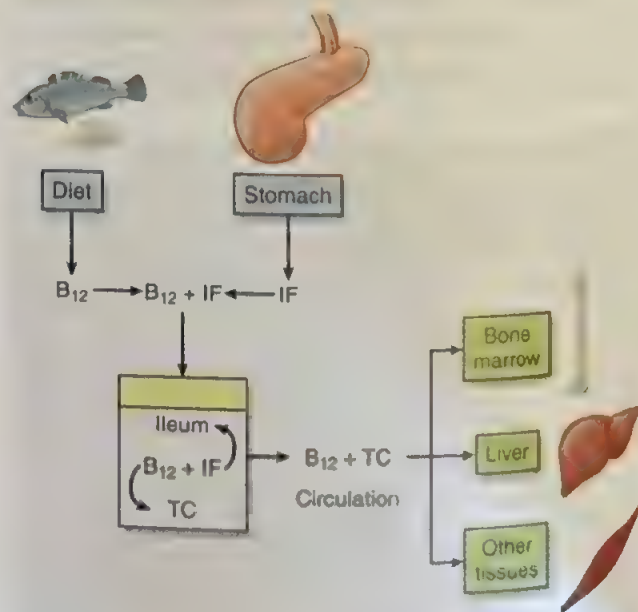


FIGURE 8-9 Transportation path of vitamin B₁₂ from the diet to the tissues. IF = intrinsic factor; TC = transcobalamin.

Immunological Factors

The serum of patients with pernicious anemia contains auto-antibodies to parietal cells, IF, and thyroid tissue. The parietal cell antibodies are found in the serum of approximately 80% to 90% of all patients with pernicious anemia. This antibody is specific for the parietal cells, but it is not specific to gastritis or pernicious anemia.^{7,18}

Antibodies to IF are demonstrated in the serum of approximately 50% to 70% of patients with pernicious anemia. These antibodies are more specific for diagnosis of pernicious anemia than antiparietal antibodies. Two types of IF antibodies have been reported. Type I, or blocking antibody, attaches to the cobalamin-binding site of IF and is found more often. Type II, or binding antibody, is detected as a complex with IF.⁷ Thyroid antibodies have often been found in the serum of patients with pernicious anemia or their relatives.^{7,18}

An association between pernicious anemia and other autoimmune diseases, such as thyroid disease, diabetes mellitus, and rheumatoid arthritis, has also been noted.⁷ The positive response to steroids in some patients with pernicious anemia supports the autoimmune mechanism.⁷

The *Helicobacter pylori* microorganism has been identified as a major cause of gastritis and peptic ulcers. This microorganism induces autoantibodies against the gastric proton pump H⁺, K⁺-ATPase in 20% to 30% of infected patients. These antibodies are associated with gastritis, increased atrophy, and apoptosis in the corpus mucosa. One current theory is that, in this way, *H. pylori* may initiate atrophic body gastritis and pernicious anemia through molecular mimicry.¹⁸

Clinical Manifestations of Vitamin B₁₂ Deficiency

The onset of pernicious anemia is generally insidious. Patients with pernicious anemia and other vitamin B₁₂ deficiencies have all the signs and symptoms of megaloblastic anemia mentioned earlier. Fever is usually present in severe anemia. Loss of appetite is a common complaint. Glossitis (sore tongue) is reported in 50% of patients.⁷ Although the initial presentations may vary among patients with vitamin B₁₂ deficiency, the classic symptoms include weakness, glossitis, and paresthesias.⁷

The bone marrow morphology of patients with vitamin B₁₂ deficiency is megaloblastic, and the peripheral smear contains macroovalocytes. In addition to hematologic abnormalities, vitamin B₁₂ deficiency is associated with gastrointestinal, thrombotic, psychiatric, and neurological complications.

Neurological Manifestations

Neurological problems are more common in pernicious anemia than in other types of vitamin B₁₂ deficiencies. The degree of neurological involvement is not directly related to the degree of anemia. Neurological manifestations can be present even in the absence of anemia or macrocytosis; one estimate is that 20% of patients with neurological signs of B₁₂ deficiency do not present with anemia.^{12,19}

The neurological abnormalities may be mild, moderate, or severe, and may involve degeneration of peripheral nerves, posterior columns of the spinal cord, and posterior and lateral columns of the spinal cord (Fig. 8-12). Because multiple neuropathies are involved, the terms *subacute combined degeneration (SCD)* and *combined system disease* may be used.^{4,7}

Cobalamin deficiency of the nervous system

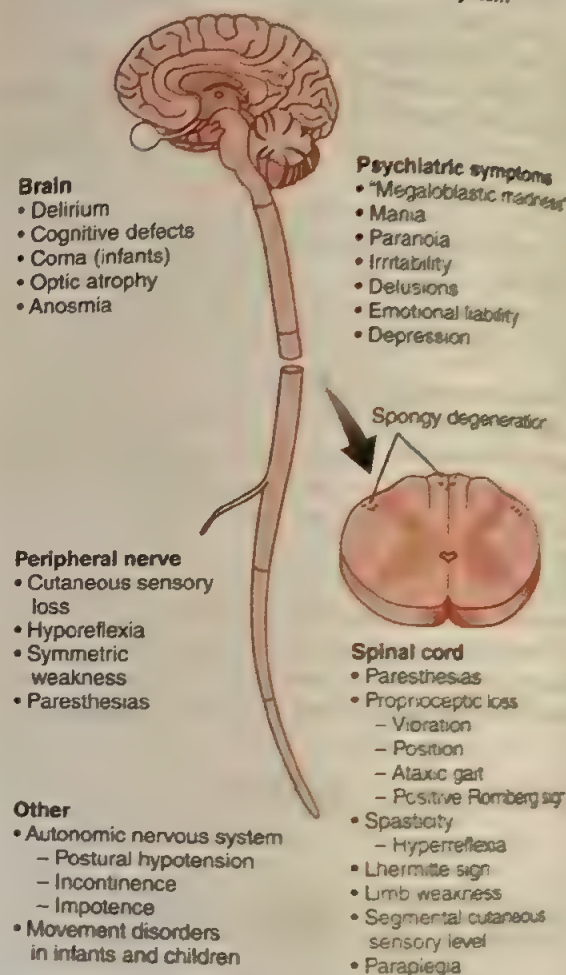


FIGURE 8-12 Neurological manifestations in pernicious anemia. (Adapted from Stabler SP. Megaloblastic anemias. In: Goldman L, Schafer AL, editors. Goldman-Cecil Medicine. 26th ed. Philadelphia: Elsevier; 2018. p. 1069-1077.e3)

ADVANCED CONTENT

Primary care practitioners often encounter subtle changes from a patient's baseline neurological, cognitive, or psychiatric findings. The most common neurological findings in vitamin B₁₂ deficiency are symmetric paresthesias or numbness and gait problems.¹² The patient often experiences symmetric tingling or "pins-and-needle" sensations in the toes and later in all four limbs. Less often, the patient may complain of numbness. At the later stage, the posterior spinal columns may be involved. At this stage, the patient may complain of clumsiness and have an incoordinate gait. The lateral spinal columns become involved in the most severe stage of illness, with manifestations of severe weakness and stiffness of limbs, impairment of memory, and depression. Severe psychiatric symptoms are less common and include stupor, hallucinations, paranoia, and severe depression. The extent of neurological recovery is unpredictable, and the

take some time. Reversal of neurological symptoms usually occurs between 6 weeks and 3 months.¹⁹ In untreated patients, the neurological symptoms are progressive, and the degree of severity is directly proportional to the duration of symptoms.⁷ Similarly, the full reversal of neurological symptoms with vitamin B₁₂ treatment is inversely related to duration of symptoms before therapy and the extent of the original dysfunction and delay in treatment.^{7,19} Still, some degree of dysfunction may remain. Residual deficits persist in 6% of patients.^{7,19}

The basic underlying cause for the neuropathy associated with vitamin B₁₂ deficiency is not exactly known. However, the impairment of methionine synthetase reaction has been indicated as the possible cause for the neuropathy. The rationale for this hypothesis is that the deficiency of methionine leads to decreased production of S-adenosyl methionine (SAM), a key intermediate in methylation reactions of myelin. The impairment of methylation in myelin could result in demyelination and, consequently, in clinical neuropathy.^{7,8}

Other Causes of Vitamin B₁₂ Deficiency

Other causes of vitamin B₁₂ deficiency include gastrectomy, blind loop syndrome, fish tapeworm, diseases of the ileum, chronic pancreatic disease, other disorders, and drug-induced vitamin deficiency (Table 8-2).

Gastrectomy

Many other causes of malabsorption can lead to vitamin B₁₂ deficiency (see Table 8-2). Gastric resection for any cause as well as gastric bypass surgery for morbid obesity can lead to multiple nutritional deficiencies. In a **gastrectomy** procedure, the IF-producing cells are removed. In the absence of vitamin B₁₂ therapy, vitamin B₁₂ deficiency develops in these patients within several years. Vitamin B₁₂ deficiency has been reported in 15% to 30% of patients with partial gastrectomy.^{2,7}

Blind Loop Syndrome In blind loop syndrome, an anatomic abnormality of the small intestine, there is an overgrowth with bacteria that thrive on vitamin B₁₂.² These microorganisms take up the vitamin B₁₂ and make it unavailable for absorption by the ileum. Antibiotic therapy reverses the malabsorption and normalizes the vitamin B₁₂ level.^{2,7}

Fish Tapeworm The classic parasitic cause of vitamin B₁₂ deficiency is due to the fish tapeworm (*Diphyllobothrium latum*). This parasite competes for vitamin B₁₂ by splitting B₁₂ from IF. The malabsorption type of vitamin B₁₂ deficiency is normally corrected when vitamin B₁₂ or B₁₂ and IF are given to the patients.^{2,7}

Diseases of Ileum Since the ileum is the site of B₁₂-IF complex absorption, vitamin B₁₂ deficiency can also be seen in diseases of the ileum. These can include ileal resection or bypass and gastrointestinal malabsorption due to inflammatory bowel disease, tropical sprue, and celiac disease.¹³ The frequency of B₁₂ malabsorption and deficiency in these states varies and often depends on the extent of ileal involvement.⁷

Chronic Pancreatic Disease In pancreatic disease, vitamin B₁₂ deficiency develops as a result of a decrease in the proteases necessary for digestion of vitamin B₁₂-binding protein haptocorrin and subsequent transfer of B₁₂ to IF for absorption.² Also a gastrinoma, a gastrin-secreting tumor in the pancreas found in Zollinger-Ellison syndrome, causes decreased uptake of B₁₂-IF complex due to the large quantity of acid preventing the binding of B₁₂ to IF.^{2,7}

Other Disorders Vitamin B₁₂ deficiency has also been reported in patients who are on hemodialysis and in patients with human immunodeficiency virus (HIV) infection and acquired immunodeficiency syndrome (AIDS).⁷

Drug-Induced Vitamin Deficiency Other causes of vitamin B₁₂ deficiency are drugs such as alcohol, anesthetics, nitrous oxide (N₂O), and the antituberculosis drug para-aminosalicylic acid (PAS).^{7,20}

TABLE 8-2 Causes of Vitamin B₁₂ Deficiency

Category	Cause
Dietary Deficiency	<ul style="list-style-type: none"> • Strict vegan diet
Malabsorption	<ul style="list-style-type: none"> • Pernicious anemia • Gastrectomy (total or partial) • Blind loop syndrome • Fish tapeworm (<i>Diphyllobothrium latum</i>) • Diseases of ileum • Chronic pancreatic disease
Other Disorders	<ul style="list-style-type: none"> • Hemodialysis • Human Immunodeficiency virus (HIV) infection • Acquired immunodeficiency syndrome (AIDS)
Drug-Induced	<ul style="list-style-type: none"> • Alcohol • Nitrous oxide (N₂O) • Para-aminosalicylic acid (PAS)

Folic Acid Deficiency

Key information concerning folic acid deficiency as an etiology of megaloblastic anemia include sources and requirements, structure, absorption and metabolism, causes of deficiency, and clinical manifestations of deficiency.

Sources and Requirements

Folic acid, also known as folate or pteroylglutamic acid, is a water-soluble vitamin present in a variety of foods. The highest concentration is present in green leafy vegetables, fruits, dairy products, cereals, and also in animal foods such as liver and kidney. The average daily diet contains about 200 to 500 mcg of folate.²¹ Unlike vitamin B₁₂, folate is not resistant to heat, and prolonged cooking of food in large quantities of water destroys folate.^{4,5,7} The recommended dietary intake of folic acid for adults is approximately 50 to 100 mcg/day.^{3,14,21} This requirement increases significantly during infancy, pregnancy, and lactation. Folate deficiency during early pregnancy (first trimester) can cause neural tube defects in the fetus and is associated with paralysis and brain damage.^{2,5,10,16} The body

storage is about 10 to 20 mg,¹⁴ of which most is stored in the liver. Folic acid is absorbed through the duodenum and jejunum, and the amount absorbed is about 60% of intake.²² Folate is lost via body secretions such as bile, urine, and sweat. Folic acid has a higher turnover time and a higher rate of loss compared with vitamin B₁₂ and, therefore, it takes only a few months to develop dietary folate deficiency.

ADVANCED CONTENT

Structure

Folic acid consists of three components: pteridine, para-aminobenzoic acid, and glutamic acid (Fig. 8-13). Folic acid derived from the diet is not biologically active. Once absorbed through the intestinal lumen, it is hydrolyzed, reduced, and methylated to form methyl tetrahydrofolate (CH₃THF). Other biologically active forms of folic acid are tetrahydrofolate (THF) and its coenzyme, N⁵,N¹⁰-methylene tetrahydrofolate (N⁵N¹⁰CH₂THF).

Serum folate is in the form of CH₃THF and enters all tissue cells in this form.

Absorption and Metabolism

Dietary folic acid is in the form of polyglutamic acid (many glutamic acid residues). Once in the intestinal lumen, it is acted on by the enzyme folate deconjugase, which is present in the epithelial cells of intestine, to form monoglutamic acid (single glutamic acid residue) (Figs. 8-14 and 8-15). Monoglutamic acid is then reduced and methylated to CH₃THF.¹⁰ When CH₃THF is absorbed from the circulation into the tissue cells, it transfers its methyl group to homocysteine to form methionine and THF. The THF formed reconstitutes with additional glutamic acid residues to form cellular THF (see Figs. 8-13 and 8-14). The THF is then methylated to form coenzyme methylene THF (N⁵N¹⁰CH₂THF) necessary for the formation of thymidine monophosphate from uridine monophosphate. This is the key reaction for DNA synthesis (see Fig. 8-10).

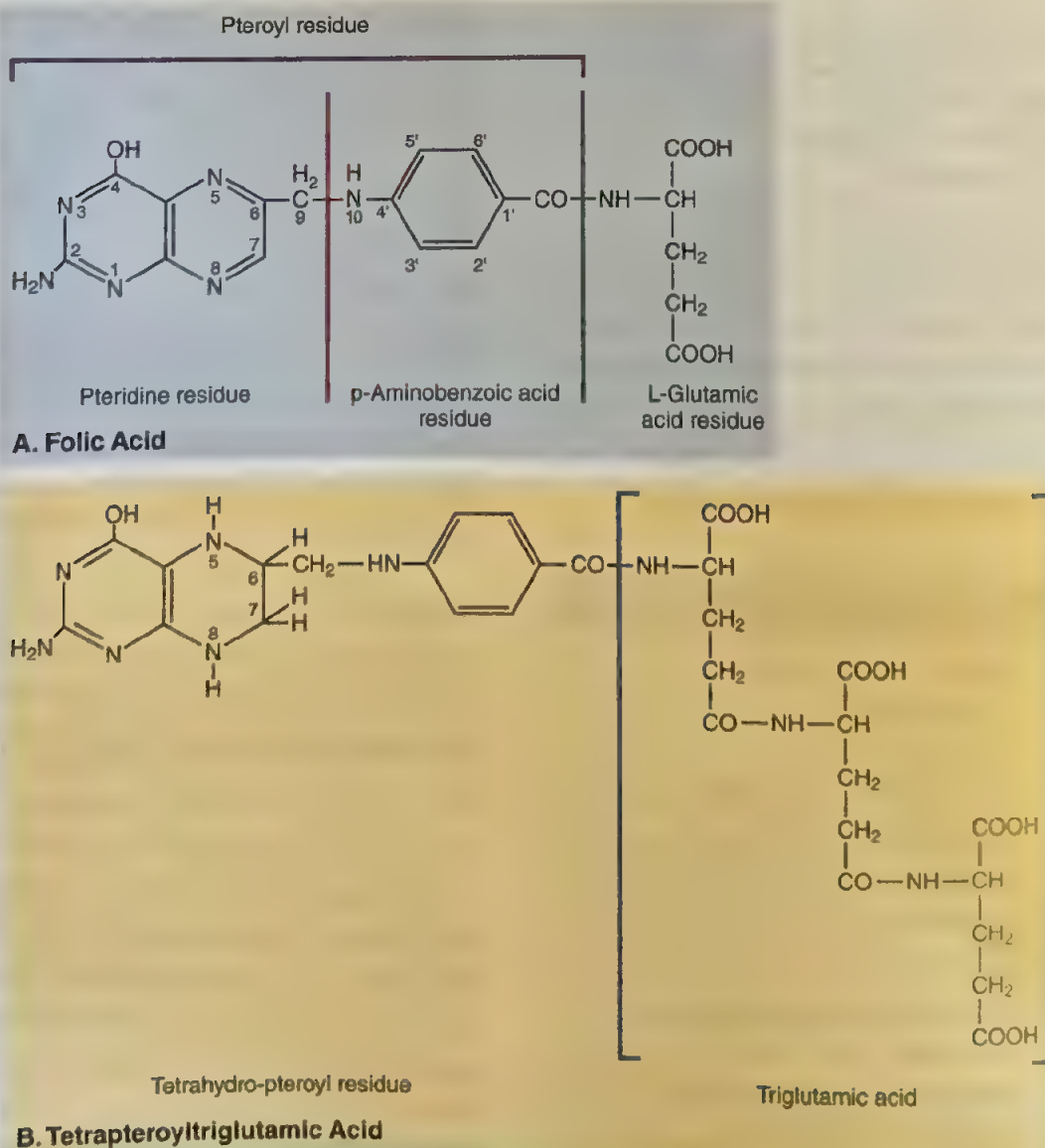


FIGURE 8-13 Structure of folic acid and its derivative. **A.** Folic acid (pteroylglutamic acid). The three components are defined by vertical lines. **B.** Tetrahydrofolate triglutamate (tetrahydrofolate triglutamate), the active form of folate present in the tissues.

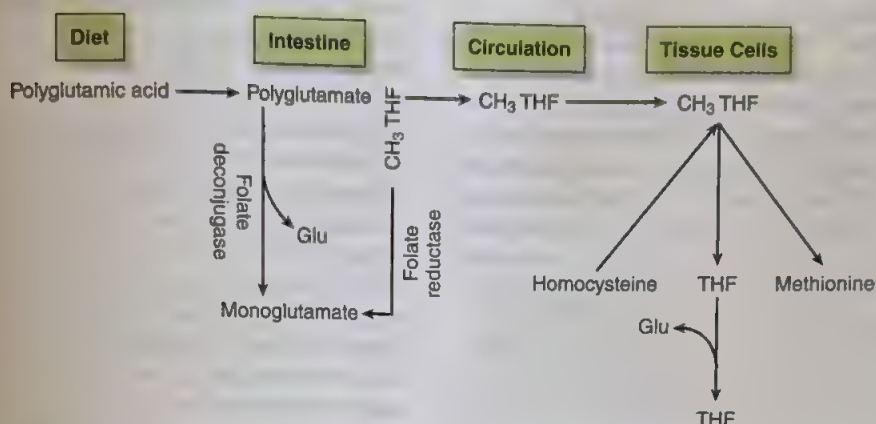


FIGURE 8-14 Absorption and metabolism of folic acid. CH₃THF – methyl tetrahydrofolate; Glu – glutamic acid; THF – tetrahydrofolate.

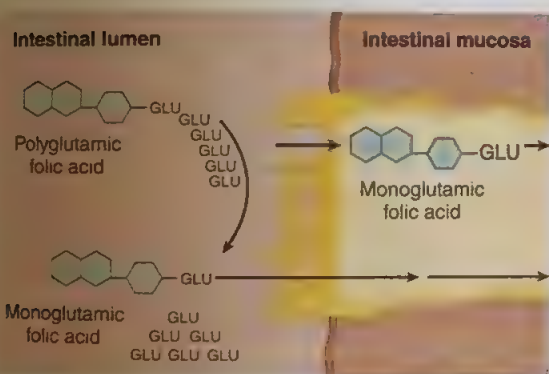


FIGURE 8-15 Intestinal absorption of the folate derivatives of food. The intestinal absorption of dietary folates is a two-step process that involves the hydrolysis of folate polyglutamates to the corresponding monoglutamyl derivatives, followed by their transport into intestinal cells. (From Streiff RR. Intestinal absorption of the folate derivatives of food JAMA 1970;214:105, with permission.)

TABLE 8-3 Causes of Folic Acid Deficiency

Category	Cause
Dietary Deficiency	<ul style="list-style-type: none"> Poverty Old age Alcoholism Chronic diseases Malabsorption Tropical sprue Gluten-sensitive enteropathy
Increased Requirement	<ul style="list-style-type: none"> Childhood celiac disease Pregnancy Infancy Malignancy Drugs Methotrexate Pyrimethamine Phenytoin Alcohol Isoniazid Oral contraceptives Others

Causes of Folic Acid Deficiency

Causes of folic acid deficiency include dietary deficiency, malabsorption, and drug-induced folate deficiency (Table 8-3).

Dietary Deficiency The main cause of folic acid deficiency is decreased dietary intake. Nutritional folate deficiency is usually a consequence of poverty, old age, alcoholism, pregnancy, and chronic diseases. In the United States, folic acid has been added to cereal grains, rice, and milled flour to increase the average adult's intake by 100 mcg.⁷ The U.S. government urges women planning to become pregnant and in early pregnancy to eat a diet rich in folic acid or take vitamin supplements to prevent the adverse effects of folic acid deficiency on their fetus.

Malabsorption The most common causes of folate malabsorption are tropical sprue and gluten-sensitive enteropathy. Tropical sprue is an infection that causes intestinal atrophy with clinical manifestations of weakness, weight loss, and steatorrhea. Tropical sprue affects the entire intestine and, therefore, causes a wide variety of nutritional deficiencies, including vitamin B₁₂ deficiency.⁷ Affected individuals respond well to antibiotics.

Gluten-sensitive enteropathy has the same clinical manifestations as those mentioned for tropical sprue. It includes both nontropical sprue and childhood celiac disease. Affected individuals cannot digest gluten, a protein found in wheat and other grains. Lesions are most severe in the proximal intestine. Childhood celiac disease is a malabsorption syndrome resulting in anemia caused by iron deficiency and, to a lesser degree, vitamin B₁₂ and folate deficiencies.

The requirement for folic acid also increases during rapid cellular proliferation in hematologic diseases such as sickle cell anemia, thalassemia, hereditary spherocytosis, and autoimmune hemolytic anemia.

Drug-Induced Folate Deficiency Drug-induced folate-deficient megaloblastic anemia has been reported with a variety of drugs such as methotrexate, pyrimethamine, phenytoin, alcohol, isoniazid, and oral contraceptives¹³ (see Table 8-3).

Clinical Manifestations of Folic Acid Deficiency

Clinical manifestations of folate deficiency are the same as those for vitamin B₁₂ deficiency, mentioned earlier. The onset of anemia is insidious, with the distinct morphology characteristic of megaloblastic anemia in the bone marrow and the peripheral blood. Although neuropathy is mainly characteristic of vitamin B₁₂ deficiency, several cases of neurological abnormalities, such as depression, dementia, and peripheral neuropathy associated with folic acid deficiency, have been reported.^{14,23} Some of these neuropathies, in particular, depression, have responded favorably to treatment with folate.^{16,23}

Folates are essential for one-carbon units transfer actions, which are important for: (1) the synthesis of purine and thymidine precursors of nucleic acids, (2) the metabolism of some amino acids, and (3) the synthesis of *s*-adenosylmethionine (SAM).²³ Folic acid and vitamin B₁₂ are both necessary cofactors of the enzyme methionine synthase, which converts homocysteine to methionine. The deficiency of these vitamins causes an increased level of plasma homocysteine. Hyperhomocysteinemia is a risk factor for thrombosis.^{1,10}

CRITICAL THINKING QUESTION

8-2 Why does folic acid deficiency develop more quickly than vitamin B₁₂ deficiency, and why is it more likely to be caused by a lack of dietary intake?

Laboratory Diagnosis of Megaloblastic Anemia

Several important factors in differential diagnosis of megaloblastic anemias are the patient's physical examination, medical history, drug history, family history, and laboratory tests.

The most common laboratory screening tests and results that are used in the diagnosis of megaloblastic anemias are low hemoglobin level, elevated MCV, and peripheral smear morphology, such as macroovalocytes and hypersegmented neutrophils. Once the diagnosis of megaloblastic anemia is established, the exact cause of the anemia should be determined for appropriate and effective treatment.

Laboratory Tests for the Diagnosis of Vitamin B₁₂ and Folic Acid Deficiencies

The most specific diagnostic tests used for vitamin B₁₂ and folate deficiencies are serum B₁₂ and serum and red cell folate levels (Table 8-4). Since serum folate levels fluctuate with dietary intake, red cell folate is a better indicator of tissue folate levels.⁶ Serum cobalamin is usually measured via chemiluminescence-based competitive-binding assays, which are normally automated.^{1,3,7,8,19,24} Serum folate may be measured by microbiological assays, radioassay, via an enzyme-linked immunosorbent assay (ELISA) or most commonly a chemiluminescent immunoassay.^{2,4,25-27}

It is worth noting that, to date, there is no standard algorithm for investigation of vitamin B₁₂ deficiency. Different laboratories choose different algorithms based on an individual patient, availability of the procedure, and the cost.

Serum B₁₂ (Serum Cobalamin) Level

The normal range for serum vitamin B₁₂ is commonly stated as 200 to 400 ng/L. In vitamin B₁₂ deficiency, the level is usually less than 200 ng/L greater than 95% of the time.¹⁴ The more severe the vitamin B₁₂ deficiency, the lower serum B₁₂ level becomes.³ Low levels of serum B₁₂ in the absence of vitamin B₁₂ deficiency have been reported in patients who are pregnant using oral contraceptives, or have folate or HC deficiency.³ Serum B₁₂ level may be normal in B₁₂ deficiency resulting from N₂O, TC deficiency, patients with inborn errors of B₁₂ metabolism, and in patients with both folate and B₁₂ deficiencies.^{5,7} Elevated serum B₁₂ is associated with myeloproliferative disorders, chronic myelocytic leukemia, and liver disease.^{5,7} Because serum B₁₂ level may be falsely low or normal, metabolic tests such as methylmalonic acid (MMA) and total homocysteine assays can be performed. The levels of these metabolites are increased before serum B₁₂ level is decreased.^{5,24}

One thing that laboratorians and physicians need to be aware of with the chemiluminescent immunoassays used for vitamin B₁₂ detection is that there is the potential for spuriously normal or elevated levels when these assays are tested against serum from patients with pernicious anemia.^{1,8,10} The false-negative results in these cases are attributed to the *in vitro* binding of anti-IF antibodies in the patient's serum to the IF found in the manufacturer's reagent.¹⁰ Most commercial assays have mitigated this problem from earlier versions of these assays, but if B₁₂ results are not concordant with the clinical presentation, this could be the cause for the discrepancy.

Serum and Red Cell Folate

Serum and red cell folate are decreased in patients with folate deficiency. The normal range for serum folate levels vary depending on the methodology used; one published range is 5 to 16 ng/mL,⁴ while an ELISA published normal range is 2 to 15 ng/mL.³ The minimal folate level for normal DNA synthesis is about 4 ng/mL.¹⁶ Serum folate is sensitive to dietary folate intake. False low serum folate levels have been reported in folate-binding protein (FBP) assays due to the different affinities that various folate forms have for FBP, and some drugs may cross-react with FBPs in an assay resulting in falsely low values.²⁵ Red cell folate is a valuable test reflecting where in the body folate stored. It is less affected by recent diet than is the serum assay. In normal adults, the red cell folate concentration ranges from 160 to 640 mcg/L of packed red cells.³ In patients with a severe B₁₂ deficiency, the serum folate level is increased while the red cell folate level is decreased. In this case, the increased serum folate level is caused by increased CH₂THF in the serum (the methyl trap hypothesis), resulting from the lack of vitamin B₁₂ necessary for conversion of homocysteine to methionine and methyl-THF to THF (see Fig. 8-10).

Other Laboratory Tests

The diagnosis of vitamin B₁₂ deficiency may be complex and inconclusive in the absence of hematologic and neurological abnormalities. Many studies have shown that some patients with vitamin B₁₂ deficiency may seek medical help because of complications other than the classical features of megaloblastic anemia. Examples of these complications

are retinopathy, neuropathy, vascular disorders, infertility, and physical and mental growth retardations in infancy.^{1,3-5,7,8,12,16,17,23,28} However, these conditions have been reversed with early vitamin B₁₂ treatment. Vitamin B₁₂ deficiency should be included in the differential diagnosis in patients with neurological and neuropsychiatric problems.^{16,23} To prevent vitamin B₁₂-associated complications, numerous attempts have been made at early diagnosis of vitamin B₁₂ and folate deficiencies.

Other laboratory tests that may support a diagnosis of vitamin B₁₂ are antibodies to IF, blood test for gastric atrophy, serum vitamin B₁₂ binding proteins, the CibaSorb test, and methylmalonic acid (MMA) and total homocysteine assays. These laboratory tests are useful when the other laboratory tests are inconclusive. Macrocytic anemia due to vitamin B₁₂ and/or folate deficiency should be distinguished from macrocytosis present in patients with myelodysplastic syndrome, hemolytic anemia, and those exposed to chemotherapeutic drugs (see Table 8-4).

Gastric Autoantibodies

Antibodies to IF are present in the serum of about 40% to 60% of patients with pernicious anemia.¹⁸ Although the IF antibody test is not a sensitive test, its specificity for the diagnosis of pernicious anemia is 95%.⁷ Decreased serum B₁₂ and the presence of the antibody to IF are indicative of pernicious anemia. Parietal cell antibody is present in 80% to 90% of patients with pernicious anemia; however, its diagnostic value for pernicious anemia is limited because this antibody is specific for immune gastritis.^{7,18}

Blood Test For Gastric Atrophy

Gastric **achlorhydria** (low gastric acidity) is present in almost all patients with pernicious anemia. Achlorhydria after histamine stimulation supports the diagnosis of pernicious anemia. However, this test is not specific, because achlorhydria has

also been reported in patients without pernicious anemia. Serum gastrin levels are increased in 80% to 90% of patients with pernicious anemia, reflecting gastric achlorhydria. However, elevated gastrin levels have many other causes such as atrophic gastritis without pernicious anemia and food-cobalamin malabsorption, which makes this test unreliable for pernicious anemia.⁷ The best test for pernicious anemia is to measure IF content in stimulated gastric juice, but this test is not widely available.⁷

Serum Cobalamin Binding Proteins

Transcobalamin is the major protein carrier of vitamin B₁₂. Cobalamin-TC complex (holoTC) is the metabolically active form of the vitamin, and the level in plasma is a sensitive measure of early vitamin B₁₂ deficiency.^{28,29} The normal level of holoTC is greater than 50 pmol/L, but it has a large window of indeterminate levels; the reference values greatly depend on the assay method used.^{28,29} When vitamin B₁₂ intake is inadequate and B₁₂ storage is depleted, the holoTC level falls below 40 pg/mL.²⁹ However, the correlation between holoTC level and depletion of vitamin B₁₂ storage is not yet fully proven, and holoTC levels have not been shown to correlate with total serum vitamin B₁₂. The method is also not approved for use in the United States, and additional research is needed to understand the mechanisms that control holoTC homeostasis in the normal population and in pathologies that alter vitamin B₁₂ transport and utilization.^{15,24}

Investigation of Absorption of B₁₂ – Cobasorb

It is important to determine whether the ability to absorb the vitamin is normal in patients who are B₁₂ deficient and IF antibody negative.²⁹ Previously, this was performed using the Schilling Test—a test based on oral administration of radioactively labeled B₁₂. The Schilling Test fell out of favor due to the exposure to radioactivity, the difficulty in performing the test, and the high-test cost. A nonisotopic B₁₂ absorption test using holoTC levels and recombinant IF has subsequently been developed, though use in the United States is limited due to the lack of FDA-cleared holoTC assays on the market.

TABLE 8-4 Laboratory Evaluation for the Diagnosis of Macrocytic Anemia

Category	Test
Screening Tests	<ul style="list-style-type: none"> Complete blood count WBC differential/RBC morphology Reticulocyte index Serum lactate dehydrogenase (LD) Iron studies Bone marrow aspirate
Vitamin B ₁₂ Deficiency	<ul style="list-style-type: none"> Serum B₁₂ level Gastric autoantibodies Blood test for gastric atrophy Serum cobalamin binding proteins Serum and urine methylmalonic acid Total homocysteine
Folate Acid Deficiency	<ul style="list-style-type: none"> Serum and red cell folate levels Serum homocysteine
Other Assays	<ul style="list-style-type: none"> CobaSorb Index of B₁₂ deficiency

ADVANCED CONTENT

The CibaSorb test for B₁₂ malabsorption is a qualitative assay based on the analysis of cyanocobalamin carried as holoTC in serum before and after oral intake of the vitamin.¹³ HoloTC concentrations and TC saturation produce a higher increase than serum B₁₂ after oral administration of three high physiological doses (9 mcg) of B₁₂. Three 9 mcg doses of B₁₂ are given orally at 6-hour intervals, and serum holoTC is measured 24 hours after the last dose is given. In patients with inherited malabsorption of B₁₂ or with IF deficiency, there are no changes in holoTC levels after the B₁₂ load, whereas in controls there are increases in holoTC levels defined as a rise in holoTC of >15% and >15 pmol/L above baseline measurement.^{13,29} The CibaSorb test is useful in detection of B₁₂ malabsorption as well as determining

whether patients will respond to low-dose B₁₂ supplements or require treatment with pharmacological doses. However, it measures only relative absorption and does not provide a quantitative estimate of bioavailability.¹³

Serum and Urine Methylmalonic Acid

Serum and urine MMA levels are both elevated in patients with vitamin B₁₂ deficiency but not folate deficiency. MMA results from accumulation of methylmalonyl Co-A. Vitamin B₁₂ in the form of adenosylcobalamin is necessary for conversion of methylmalonyl CoA to succinyl CoA (see Fig. 8–11). In the absence of vitamin B₁₂, the level of methylmalonyl CoA is increased in the serum and consequently in the urine. Falsely elevated results have been reported in non-vitamin-deficient patients with renal disease, inborn errors of metabolism, and bacterial infection.⁷

It is widely acknowledged that there is no single best biomarker for the diagnosis of B₁₂ deficiency,^{3,5,7,15,29,30} and it is also agreed that there is no real gold standard laboratory marker of vitamin B₁₂ status.^{8,14,24,30} However, measurement of serum and urinary MMA is a reliable indicator of tissue vitamin B₁₂ deficiency in the absence of decreased serum B₁₂ and lack of clinical manifestations of megaloblastic anemia.^{3,5,7,29}

Normal individuals excrete 0 to 3.4 mg MMA per day. In patients with B₁₂ deficiency, MMA excretion is usually elevated.⁵ The sensitivity of a serum MMA value higher than 400 nmol/L is about 98% for clinical vitamin B₁₂ deficiency, but specificity for clinical abnormalities is much less due to reasons previously discussed.¹ The normal range for serum MMA is 60 to 360 ng/mL.³¹ This level increases to 2 to

100 times the upper limit of normal in patients with vitamin B₁₂ deficiency. However, serum MMA assay is complex, expensive, and not available in most routine clinical laboratories due to the common methodology being a liquid chromatography tandem mass spectrometry.²⁹

Serum Homocysteine

Serum homocysteine (Hcy) levels are elevated in patients with both vitamin B₁₂ and folate deficiencies, because both vitamins are necessary in the conversion of homocysteine to methionine. Serum homocysteine is also increased in a short-term folate-restricted diet and in patients with congenital homocystinuria and vitamin B₆ inhibitor.^{7,25–27}

ADVANCED CONTENT

Index of B₁₂ Deficiency

Due to the importance of timely diagnosis and treatment of B₁₂ deficiency, there have been several studies performed to try to improve the diagnostic accuracy of the available laboratory assays used for diagnosis of B₁₂ deficiency. There has been a trend to combine the use of two or more of the biomarkers into a combined indicator of B₁₂ status,² and one new approach described by Fedosov and colleagues uses all four biomarkers—B₁₂, holoTC, MMA, and Hcy—combined to determine and index of B₁₂ deficiency (4cB12).³⁰ Due to the high cost of performing all four tests, the current recommendation for investigation of B₁₂ deficiency remains a stratified, step-wise, or algorithmic approach (see Fig. 8–16).³⁰

Diagnostic testing for suspected vitamin B12 or folate deficiency

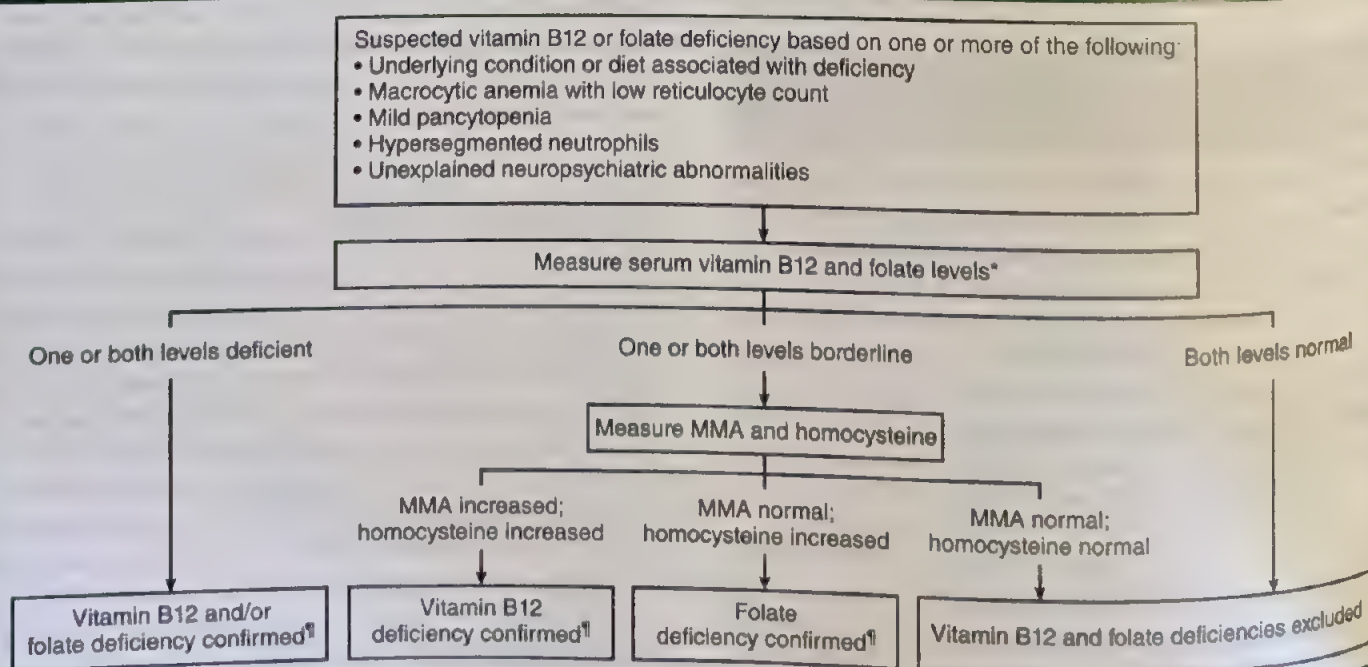


FIGURE 8-16 Algorithm for diagnostic testing for suspected vitamin B₁₂ or folate deficient patients. Adapted from Means RT, Fairfield KM. Clinical manifestations and diagnosis of vitamin B₁₂ and folate deficiency. UpToDate [Internet]. Wolters Kluwer; c2020 [updated 2020 Apr 28; cited 2020 May 28].

Treatment

Transfusion is rarely required in patients with megaloblastic anemia unless the anemia is severe and symptomatic or is associated with congestive heart failure. In these cases, packed red cells should be administered slowly to prevent pulmonary edema.⁵

Therapy for Vitamin B₁₂ Deficiency

Most people with a vitamin B₁₂ deficiency require lifelong vitamin therapy. Cyanocobalamin and hydroxocobalamin are the two therapeutic forms of vitamin B₁₂. Hydroxocobalamin is preferred by some physicians because it has a longer half-life. Cyanocobalamin is less expensive and converts to the physiological form.

Vitamin B₁₂ is injected intramuscularly or subcutaneously. Although the treatment protocol varies, the initial dose administered is higher to saturate the body storage. Vitamin B₁₂ may be given as 100 to 1,000 mcg/day for 2 weeks, then weekly until hematologic values are normalized, and then monthly for life.⁵ Vitamin B₁₂ therapy can be monitored by reticulocyte counts.

ADVANCED CONTENT

Vitamin B₁₂ can also be administered orally to patients with dietary vitamin deficiency or to those who cannot tolerate parenteral treatment. Several studies have shown that oral vitamin therapy can be as effective and efficient as parenteral therapy in conducting hematologic and neurological remissions and in maintaining normal serum B₁₂.^{13,32} In patients with pernicious anemia, about 1% of the oral dose is absorbed.⁵ Therefore, high doses of oral vitamin (1,000 to 2,000 mcg/day) are sufficient to replace the parenteral therapy. The efficiency of the treatment should be checked by occasional measurement of serum B₁₂ in patients undergoing the oral therapy.^{5,32}

Therapy for Folic Acid Deficiency

The recommended therapeutic dose to treat folate deficiency is 1 to 5 mg/day for 2 to 3 weeks.^{5,7} Folic acid vitamin is water soluble and is given orally. Lifelong therapy is not required because it is usually possible to treat folate deficiency within a short period of time.^{5,14} Folic acid may be given along with vitamin B₁₂ when both vitamins are deficient or in a therapeutic trial when the exact cause of megaloblastic anemia is not known. In this case, folate therapy will correct the hematologic abnormalities in vitamin B₁₂ patients, but the neurological manifestations will progress.

Folic acid given as prophylaxis (0.25 to 0.5 mg/day) is recommended during pregnancy and dialysis and may be required in patients with hemolytic anemia and in patients who are on antifolate drugs.⁵ Folic acid can be injected to hospitalized patients and in those who cannot take the medication by mouth.

Response to Therapy

The initial sign of a positive response to therapy is an increase in the reticulocyte count. The number of circulatory reticulocytes increases 3 to 5 days after therapy, with a peak at about 4 to

10 days.^{5,7} The megaloblastic morphology of the bone marrow disappears within 24 to 48 hours after therapy. The hematocrit rises in about 5 to 7 days after therapy, reaching normal levels in 4 to 8 weeks. Giant metamyelocytes and hypersegmented neutrophils disappear within 2 weeks. The entire therapeutic response process may take only 3 to 6 weeks, depending on the severity of the disease.¹⁹

Macrocytic Nonmegaloblastic Anemias

Macrocytic anemias may be megaloblastic or nonmegaloblastic. Differentiation between the two is important. In macrocytic, normoblastic anemias, the MCV is more than 100 fL but not as high as in megaloblastic anemias (where the MCV is more than 110 fL). The red cells on the peripheral blood smear appear large and round but not oval. The neutrophils are not hypersegmented. The bone marrow is normocellular or hypercellular with erythroid hyperplasia. The red cell precursors in the marrow are normoblastic and not megaloblastic. The mechanism responsible for the macrocytic morphology may be associated with an increase in both red cell membrane cholesterol and phospholipid. Increased lipid deposition onto the red cell membrane and altered maturation time of the red cell precursors are among the possible causes.^{33,34}

The most common causes of macrocytic anemia are chronic liver disease and alcoholism. In 40% to 96% of alcoholics, macrocytosis is present in the absence of anemia, and in this case, alcohol has a direct toxic effect on the red cells rather than causing folate deficiency. The finding of macrocytosis is a valuable screening test for early detection of alcoholism.⁷ Liver function tests such as serum LD and bilirubin are helpful in the diagnosis.

Macrocytic anemia with an elevated reticulocyte count is associated with hemolytic anemia or acute blood loss. Macrocytic anemia may also be associated with aplastic anemia, chronic myeloproliferative disorders, and sideroblastic anemia. In sideroblastic anemia, a dimorphic red cell population may be observed. Bone marrow examination is often required for differential diagnosis (see Table 8-4).

Macrocytosis has also been reported in patients taking immunosuppressive drugs as well as arsenic and chlordane intoxication. Causes of macrocytic nonmegaloblastic anemia are summarized in Box 8-1.

BOX 8-1 Causes of Macrocytic Nonmegaloblastic Anemias

- Chronic liver disease
- Alcoholism
- Acute hemorrhage
- Hypothyroidism
- Hematologic disorders
- Hemolytic anemia
- Aplastic anemia
- Chronic myeloproliferative disorders
- Immunosuppressive drugs
- Arsenic and chlordane intoxication

▶ ADVANCED CONTENT

Vitamin-Independent Megaloblastic Changes

Vitamin-independent megaloblastic changes occur in a group of disorders characterized by the presence of megaloblastic changes in the bone marrow and in the peripheral blood that are refractory to vitamin B₁₂ and folic acid treatments. The megaloblastic changes in this group of patients may occur because of an inherited or acquired predisposition, or they may be drug induced (Table 8-5).

Inherited

Orotic aciduria is a rare inherited disorder of pyrimidine metabolism characterized by increased excretion of orotic acid in the urine. Lesch-Nyhan syndrome is an X-linked

TABLE 8-5 Vitamin-Independent Megaloblastic Anemias

Type	Examples
Inherited	<ul style="list-style-type: none"> • Orotic aciduria • Lesch-Nyhan syndrome • Dihydrofolate reductase deficiency • Methyl tetrahydrofolate transferase deficiency • Transcobalamin deficiency • Abnormal cobalamin molecule • Congenital IF deficiency • Methylmalonic aciduria • Homocystinuria • Congenital dyserythropoiesis • Grasbeck syndrome
Acquired	<ul style="list-style-type: none"> • Myelodysplastic syndrome • Erythroleukemia
Drug-induced	<ul style="list-style-type: none"> • Folate antagonist (methotrexate) • Purine or pyrimidine antagonists (6-mercaptopurine, cytosine arabinoside, and hydroxyurea) • Nitrous oxide • Azidothymidine (AZT) • Others
Toxic Materials	<ul style="list-style-type: none"> • Arsenic • Chlordane • Others

disorder of purine metabolism. Megaloblastic changes are also noted in inherited dihydrofolate reductase deficiency, a disorder of folate metabolism and methyl tetrahydrofolate transferase. Inherited disorders of transcobalamin deficiency or abnormal transcobalamin molecule and congenital IF deficiency are associated with megaloblastic anemia. In congenital dyserythropoiesis, the megaloblastic changes are limited only to erythrocyte precursors of the bone marrow and red cells in the peripheral smear. White blood cells and platelets are normal. Hereditary juvenile megaloblastic anemia is caused by intestinal malabsorption of vitamin B₁₂. Grasbeck syndrome (IGS) is associated with total absence of vitamin B₁₂ absorption that is not corrected by the administration of IF.^{2,8} There are other inherited disorders that are associated with methylmalonic aciduria, homocystinuria, or both. In these disorders, pancytopenia is present with or without megaloblastic pictures.⁷

Acquired

Megaloblastic changes may be secondary to hematologic disorders such as myelodysplastic syndromes and erythroleukemia. Megaloblastic anemia may also be associated with a variety of drugs and toxic material.²⁰

Drug and Toxin Induced

A wide variety of drugs with differing modes of action are associated with megaloblastic anemia. Examples of these drugs are methotrexate, hydroxyurea, and aminopterin. They are inhibitors of dihydrofolate reductase, preventing dihydrofolate to tetrahydrofolate reaction. This inhibition results in decreased thymidine biosynthesis and DNA maturation. Drugs such as 6-mercaptopurine, cytosine arabinoside, and hydroxyurea interfere with purine or pyrimidine metabolism. Nitrous oxide (N₂O), an anesthetic gas, inactivates vitamin B₁₂.^{5,7,11,15} Azidothymidine (AZT) is associated with megaloblastic changes. Exposure to toxic materials can also cause megaloblastic anemia.⁵

CRITICAL THINKING QUESTION

8-3 Why is it important to differentiate between a vitamin-deficient diet, a process involving malabsorption, and other causes of anemia when evaluating a patient who presents with a macrocytic and/or megaloblastic anemia?

SUMMARY CHART

- Megaloblastic anemia is a macrocytic anemia characterized by defective nuclear maturation caused by impairment of DNA synthesis.
- Clinical manifestations that are often associated with vitamin B₁₂ deficiency are anemia, fever, glossitis, and neurological symptoms.
- Clinical manifestations associated with folic acid deficiency are similar to those in vitamin B₁₂ deficiency, with neuropathies not being the prominent features.
- Megaloblastic anemia is associated with ineffective erythropoiesis, ineffective granulopoiesis, and ineffective thrombopoiesis.
- The bone marrow of patients with megaloblastic anemia is hypercellular with a low M:E ratio (1:1 to 1:3) and a high number of abnormal megaloblasts, as well as abnormal granulocyte and platelet precursor cells.
- The peripheral blood is characterized by pancytopenia, macrocytes, macroovalocytes, and hypersegmented neutrophils.
- IF binds to vitamin B₁₂ and forms B₁₂-IF complex, which allows vitamin B₁₂ to be absorbed through receptors present in the ileum.
- Folate and homocysteine are required for the absorption and metabolism of folic acid.
- The major causes of megaloblastic anemias are vitamin B₁₂ deficiency, folic acid deficiency, or both.
- The main cause of vitamin B₁₂ deficiency is pernicious anemia, a progressive autoimmune disorder affecting intrinsic factor's ability to absorb B₁₂ in the intestine.
- Other causes of vitamin B₁₂ deficiency are dietary malabsorption secondary to diseases and drugs.
- The main cause of folic acid deficiency is a poor diet; other causes of folic acid deficiency are malabsorption, increased requirement, and drugs.
- The main difference between pernicious anemia and other malabsorption-related causes for vitamin B₁₂ deficiency is the presence of autoantibodies to parietal cells, IF, and thyroid tissue in the serum of patients with pernicious anemia.
- Pregnant women, lactating women, growing children, and the elderly are all at risk for vitamin B₁₂ and folic acid deficiency.
- Vegetarians and vegans and infants being breastfed by a vegan or B₁₂-deficient mother are at particular risk for vitamin B₁₂ deficiency.
- Individuals that suffer from poverty, alcoholism, or chronic disease are more likely to experience folic acid deficiency.
- Laboratory tests used for the differential diagnosis of vitamin B₁₂ and folate deficiencies are serum B₁₂, and serum and red cell folate.
- Other laboratory tests that may be useful are gastric achlorhydria, antibodies to intrinsic factor, and serum and urine methylmalonic acid, serum and urine homocysteine.
- Most people with a vitamin B₁₂ deficiency require lifelong vitamin therapy.
- Lifelong therapy is not required for folic acid deficiency because it is usually possible to treat within a short period of time.
- Vitamin-independent megaloblastic changes occur in a group of disorders characterized by the presence of megaloblastic changes in the bone marrow and in the peripheral blood that are refractory to vitamin B₁₂ and folic acid treatments.
- The most common causes of macrocytic nonmegaloblastic anemia are liver disease and alcoholism.

CASE STUDY 8-1

REASON FOR VISIT: A 50-year-old woman visited her physician because she had experienced weakness, fatigue, and shortness of breath for the past few months.

PATIENT AND FAMILY MEDICAL HISTORY: Her family history and her past medical history were unremarkable. The patient reports eating a balanced diet and is not a vegetarian or vegan. She is not pregnant or breastfeeding.

MEDICATION HISTORY: The patient was not taking any medications.

PHYSICAL EXAMINATION FINDINGS: Physical examination revealed a tall, slender woman with "lemon-yellow skin" and a smooth, red tongue. No hepatosplenomegaly was noted. She had experienced a loss of vibratory sense and had some problems with gait coordination.

Continued

CASE STUDY 8-1—cont'd

LABORATORY RESULTS:

Laboratory Test	Result	Reference Ranges
CBC		
RBC	$2.2 \times 10^6/\mu\text{L}$	$4.1\text{--}5.1 \times 10^6/\mu\text{L}$
Hb	8.5 g/dL	12.3–15.3 g/dL
Hct	25%	36–45%
MCV	114 fL	87–98 fL
MCH	38.6 pg	27–35 pg
MCHC	34%	32–36%
PLT	$170 \times 10^3/\text{L}$	$150\text{--}450 \times 10^3/\mu\text{L}$
WBC	$7.4 \times 10^3/\text{L}$	$4.4\text{--}11 \times 10^3/\mu\text{L}$
Neutrophils	67% segmented neutrophils (5% hypersegmented)	60%–70%
Lymphocytes	25%	20%–30%
Monocytes	5%	5%–10%
Eosinophils	3%	0%–5%
RBC Morphology		Macrocytic with few macroovalocytes and few schistocytes. RBC inclusions: few Howell–Jolly bodies.
Reticulocyte Count		0.2% (N: 0.5 to 2%)
Chemistry Test		
Bilirubin	4.0 mg/dL	0.2–1.2 mg/dL
Indirect bilirubin	2.9 mg/dL	0.2–0.8 mg/dL
Serum iron	220 mcg/dL	50–170 mcg/dL
LD	720 U/L	100–250 U/L
TIBC	215 mcg/dL	250–450 mcg/dL
Serum B12	50 pg/mL	100–700 pg/mL
Red cell folate	200 ng/mL	140–628 ng/mL

QUESTIONS

- Based on the CBC results, would you describe this patient as anemic?
- After reviewing differential and chemistry findings and taking into account her physical findings, which type of anemia is likely present in this patient?
- What does the decreased reticulocyte count in this patient represent?
- What are the most common causes of this type of anemia?
- Based on this patient's laboratory findings, which cause is likely the reason for her diagnosis?
- What test(s) could be ordered as follow-up to distinguish this patient's specific cause for her diagnosis?

ANSWERS

- Yes. The CBC revealed a low RBC count, low Hgb, and low Hct, all contributing to a diagnosis of anemia.
- The diagnosis of megaloblastic anemia can be made based on the patient's physical examination and the highlights of the laboratory results. Physical findings

reveal that the patient had the signs and symptoms of anemia, with the “lemon-yellow skin” caused by anemia and jaundice. The low hemoglobin and hematocrit levels in combination with an elevated MCV and normal MCHC are suggestive of macrocytic, normochromic anemia, a characteristic of megaloblastic anemia. The macroovalocytes and hypersegmented neutrophils present on the peripheral smear are striking features of megaloblastic anemia. Howell–Jolly bodies are the red cell inclusions commonly seen in this type of anemia. The low reticulocyte value is indicative of ineffective erythropoiesis, and the low serum B₁₂ points to megaloblastic anemia. The white blood cell (WBC) and platelet counts are normal in this patient, as is expected in early stages of megaloblastic anemia.

The elevated bilirubin, serum LDH, and serum iron levels can all be attributed to red cell hemolysis. In megaloblastic anemia, the red cells are fragile and have a shortened life span, and are thus prematurely destroyed in the bone marrow and in the peripheral blood.

- This patient has a decreased reticulocyte count, which indicates ineffective erythropoiesis. Patients with megaloblastic anemia are expected to have decreased reticulocyte production indexes of less than 2.
- The most common causes of megaloblastic anemia are vitamin B₁₂ and folate deficiencies.
- In this patient, the serum B₁₂ level was decreased, whereas the red cell folate level was normal. These findings indicate that B₁₂ deficiency was the probable cause of the megaloblastic anemia. Since the patient was eating a balanced diet and had no past medical history, it is likely that her vitamin B₁₂ deficiency is caused by malabsorption.
- Testing for autoantibodies to intrinsic factor and parietal cells should be ordered to evaluate the specific cause of B₁₂ malabsorption. If the anti-intrinsic factor antibody and antiparietal cell antibody tests are positive for this patient, pernicious anemia can be diagnosed.

CASE CONTINUATION

The patient tested positive for both anti-intrinsic factor and antiparietal antibodies and was therefore put on hydroxocobalamin therapy. The initial doses of hydroxocobalamin were administered intramuscularly for several weeks, followed by maintenance doses. After 2 months of therapy, the patient had completely recovered. Her hemoglobin and hematocrit had returned to normal, and her abnormal blood cell morphologies had disappeared. The patient was advised to continue the vitamin B₁₂ therapy to prevent relapse of the symptoms, because lack of IF in patients with pernicious anemia prevents absorption of dietary B₁₂.

N = normal range.

CASE STUDY 8-2

REASON FOR VISIT: A 12-year-old girl presented with complaints of yellowish discoloration of eyes and palms and loss of appetite associated with weight loss for the past 6 months. She also reported easy fatigability for the past 2 weeks.

PATIENT AND FAMILY MEDICAL HISTORY: She had no history of abdominal pain, pale stools, or bone pain. She had no history of substance abuse, blood transfusions, or drug intake. Parents reported that she has been on a vegetarian diet for the past 2 years. Her birth history, developmental history, and family history were unremarkable.

MEDICATION HISTORY: The patient had not been taking any prescribed or over-the-counter medications.

PHYSICAL EXAMINATION FINDINGS: Physical examination showed icterus, mild pallor, normal liver span, and no splenomegaly.

LABORATORY RESULTS:

CBC		
Laboratory Test	Result	Reference Ranges
RBC	$2.37 \times 10^6/\mu\text{L}$	4.2–5 μL
Hb	9.4 g/dL	12.3–15.3 g/dL
Hct	28.3%	36%–45%
MCV	119.5 fL	87–98 fL
RDW	16.7%	<15%
MCH	40.9 pg	32–36 pg
MCHC	33.2 g/dL	33.4–35.5 g/dL
PLT	$152 \times 10^3/\text{L}$	$150\text{--}450 \times 10^3/\mu\text{L}$
WBC	$5.7 \times 10^3/\mu\text{L}$	$4.4\text{--}11 \times 10^3/\mu\text{L}$
Neutrophils	80% with hypersegmentation	60%–70%
Lymphocytes	15%	20%–30%
Monocytes	5%	5%–10%
Reticulocytes	2.25%	0.5%–1.5%
Chemistry Test		
Bilirubin, total	7.5 mg/dL	0.1–1.2 mg/L
Bilirubin, direct	0.6 mg/dL	less than 0.3 mg/dL
Serum vitamin B ₁₂	<30 pg/mL	100–700 pg/mL
LD	729 IU/L	100–190 IU/L

Laboratory results included the CBC and Chemistry results shown in the table above. Additionally, the patient had normal liver enzymes and normal random blood sugar. Peripheral smear showed macrocytosis. Folate levels and iron studies were normal.

Repeat investigations, after 6 months of oral therapy with vitamin B₁₂ (1500 mcg/day), persisted to show macrocytosis, hypersegmented neutrophils, borderline unconjugated bilirubinemia, normal hemoglobin, and improving vitamin B₁₂ levels (82.3 pg/mL).

QUESTIONS

1. What is likely causing this patient's vitamin B₁₂ deficiency?
2. Would you expect this particular patient to have a full therapeutic response to the vitamin therapy after 6 months?

ANSWERS:

1. The patient's history revealed that she had been following a vegetarian diet for the past 2 years. Vegetarians are an at-risk population for vitamin B₁₂ deficiency.
2. Yes. Replacement with vitamin B₁₂ should improve most laboratory values and reverse the anemia caused by the deficiency, since the deficiency is not caused by a malabsorption issue, but rather a nutritional deficit.

CASE CONTINUATION

The 6-month follow-up visit revealed that the patient's compliance with her medication regimen was poor, and she had also resumed her vegetarian diet. Monthly injectable vitamin B₁₂ was offered to the patient, but it was declined. Oral drug compliance was ensured by counseling the patient and family.

Ten months after the first presentation, the patient reported significant improvement in well-being, and repeat investigations showed normal serum vitamin B₁₂ levels and a weight gain. Laboratory results at this time showed hemoglobin, reticulocyte count, bilirubin, LDH, and serum vitamin B₁₂ within normal limits, as well as the return of normal RBC and neutrophil morphology on peripheral smear. This symptom reversal is achievable in a majority of patients with deficiency due to nutritional effects. As with many disease states, recovery in vitamin deficient states relies on patient compliance to the treatment regimen. Compliance can often be achieved through patient and family education about the disease severity and probability of recurrence of symptoms due to poor adherence.³⁵

Adapted from: Katakam PK, Hegde AP, Venkataramaiahya M. Vitamin B₁₂ deficiency: unusual cause of jaundice in an adolescent. *BMJ Case Rep.* 2018; 2018: bcr2017222302. doi: 10.1136/bcr-2017-222302

CASE STUDY 8-3

REASON FOR VISIT: A 57-year-old nonvegetarian man presented with a 3-week history of progressive generalized weakness and dyspnea on exertion.

PATIENT AND FAMILY MEDICAL HISTORY: He had a previous history of alcohol abuse about 3 years earlier.

MEDICATION HISTORY: The patient is taking over-the-counter NSAIDs as needed for occasional muscle pain. No prescribed medications reported.

PHYSICAL EXAM FINDINGS: Physical examination was only significant for pallor.

LABORATORY RESULTS:

CBC		
Laboratory Test	Result	Reference Ranges
RBC	$1.5 \times 10^6/\mu\text{L}$	$4.0\text{--}5.0 \times 10^6/\mu\text{L}$
Hb	4.3 g/dL	13.0–17.0 g/dL
Hct	21%	36%–45%
MCV	102 fL	80–94 fL
MCH	28.7 pg	27–35 pg
MCHC	28.7 %	32%–36%
RDW	16.2%	<15%
PLT	$92 \times 10^3/\mu\text{L}$	$150\text{--}450 \times 10^3/\mu\text{L}$
WBC	$3.4 \times 10^3/\mu\text{L}$	$4.5\text{--}10.8 \times 10^3/\mu\text{L}$
Neutrophils	73.2%	60%–70%
Lymphocytes	21%	20%–30%
Monocytes	1.7%	5%–10%

Laboratory results included the CBC results shown in the table above. His hemoglobin 2 weeks before this presentation was 9 g/dL. A basic metabolic panel and coagulation studies were within normal limits. Direct antiglobulin test was negative, thyroid-stimulating hormone was normal, and a hepatitis panel, stool for occult blood, and HIV screen were negative.

QUESTION

- Based on these laboratory findings, would you classify the patient's severe anemia as megaloblastic at this time? Why or why not?

ANSWER

- No. While the patient is severely anemic, the MCV is not higher than 110 fL, the differential examination does not present with hypersegmented neutrophils, and the lack of autoantibodies that would be detected in the direct antiglobulin test rules out pernicious anemia. However, further laboratory studies to investigate this patient's case should include reticulocyte index, haptoglobin, bilirubin, and LDH to complete the investigation of a hemolytic anemia. Serum vitamin B₁₂, serum folate, serum iron and total iron-binding capacity, and ferritin should be included to evaluate the patient's vitamin status.

CASE CONTINUATION

Due to his severely anemic state, the patient received two units of packed red blood cells. The practitioner also started the patient on vitamin therapy for vitamin B₁₂ at 1,000 mcg/day. However, his pancytopenia continued to worsen.

Further laboratory studies were conducted to investigate this patient's case. Those studies with their results are as follows:

Test	Result	Reference Ranges
Reticulocyte index	0.16	0.5–2.5
Haptoglobin (mg/dL)	<8	30–200 mg/dL
Total bilirubin (mg/dL)	2.3	0–1.5 mg/dL
Direct bilirubin (mg/dL)	1.2	<0.3 mg/dL
LDH (U/L)	2510	100–220 U/L
Serum vitamin B ₁₂ (pg/mL)	68	200–700 pg/mL
Serum folate (ng/mL)	7.8	>4 ng/mL
Ferritin	110	12–300 ng/mL
Total iron-binding capacity (mcg/dL)	325	240–450 mcg/dL
Anti-IF antibodies	NEG	
Antiparietal cell antibodies	NEG	

Since the patient's pancytopenia did worsen despite the patient's transfusion and B₁₂ therapy, a bone marrow biopsy was performed. The preliminary biopsy reported revealed an elevated blast percentage, and the patient was transferred to a tertiary care center for further evaluation. There, a repeat biopsy revealed 90% cellularity with trilineage hematopoiesis and was notable for numerous giant band forms, left-shifted erythroid hyperplasia, and dyspoietic features in all three lineages.

QUESTION

- Do these additional findings change whether you view this patient's anemia as megaloblastic or not? Why?

ANSWER

- This additional information is able to explain that the patient is vitamin B₁₂ deficient, and despite the lower MCV and normal MCH, the clinical information supports a diagnosis of megaloblastic anemia. This patient does illustrate a case of vitamin B₁₂ deficiency with macrocytosis, pancytopenia, and concurrent hemolysis. The patient has a low reticulocyte count, indicating an inadequate bone marrow response to the anemia. Hemolysis in patients with B₁₂ deficiency has been attributed to intramedullary destruction of red blood cells due to ineffective erythropoiesis.

CASE CONTINUATION

The patient received daily parenteral vitamin B₁₂ injections with rapid bone marrow recovery, as evidence by improvement in the blood counts and reticulocyte index.

CASE STUDY 8-3—cont'd

He was discharged 6 days later and continued to receive vitamin B₁₂ injections once weekly as an outpatient. Upon 45-day follow-up in the clinic, laboratory revealed improvement in all cell counts, resolution of macrocytosis, and return of LDH to a normal level. This patient illustrates a case of vitamin B₁₂ deficiency with macrocytosis, pancytopenia, and concurrent hemolysis. Hemolysis in patients with B₁₂ deficiency has been attributed to intramedullary destruction of red blood cells due to ineffective erythropoiesis. The patient's previous history of alcohol abuse combined with chronic malabsorption could have contributed to this deficiency. This could also explain the initial lack of response to oral vitamin B₁₂ supplementation but great response with complete normalization of cell counts with parenteral supplementation. His B₁₂ <200 pg/mL along with clinical evidence of disease is consistent with deficiency, and no further confirmatory testing is needed. Rather, it is in cases where B₁₂ levels range from 200 to 300 pg/mL where deficiency is possible, and verification with serum methylmalonic acid

and/or serum homocysteine levels may be necessary. Finally, this case is important to recognize that bone marrow biopsy in patients with B₁₂ deficiency can be misleading since it may appear to show dysplastic changes mimicking myelodysplastic syndrome or acute leukemia.

QUESTION

3. What could be the cause of the patient's vitamin B₁₂ deficiency?

ANSWER

3. The patient's previous history of alcohol abuse combined with poor nutrition or chronic malabsorption could have contributed to this deficiency of B₁₂. Pernicious anemia is unlikely due to the lack of anti-IF and antiparietal cell antibodies.

Adapted from Konda M, Godbole A, Pandey S, Sasapu A. Vitamin B₁₂ deficiency mimicking acute leukemia. *Proc (Bayl Univ Med Cent)*. 2019;32:589-592. doi: 10.1080/08998280.2019.1641045

REVIEW QUESTIONS

- The pathophysiology of megaloblastic anemia is:
 - Defective RNA synthesis and abnormal cytoplasm maturation
 - Defective DNA synthesis and abnormal nuclear maturation
 - Defective RNA synthesis and abnormal nuclear maturation
 - Defective DNA synthesis and abnormal cytoplasm maturation
- Both B₁₂ deficiency and folate deficiency present with which clinical manifestation?
 - Anemia
 - Thrombocytosis
 - Hemoglobinemia
 - Leukocytosis
- What is the hallmark of megaloblastic anemia?
 - Normocytic normochromic anemia
 - MCV greater than 100 fL
 - Leukocytosis
 - Thrombocytosis
- Which of the following are the characteristic findings of the bone marrow in patients with megaloblastic anemia?
 - Hypocellular with low M:E ratio
 - Hypercellular with high M:E ratio
 - Hypocellular with high M:E ratio
 - Hypercellular with low M:E ratio
- Which of the following is a non-red blood cell finding seen on the peripheral smear of a patient with megaloblastic anemia?
 - Macroovalocytes
 - Hypersegmented neutrophils
 - Schistocytes
 - Howell-Jolly bodies
- According to the morphological classification of anemias, megaloblastic anemia is a:
 - Macrocytic, hypochromic anemia
 - Macrocytic, hyperchromic anemia
 - Macrocytic, normochromic anemia
 - Normocytic, normochromic anemia
- The glycoprotein necessary for absorption of vitamin B₁₂ is:
 - Albumin
 - Transcobalamin
 - Haptoglobin
 - Intrinsic factor
- All of the following are causes of B₁₂ deficiency, except:
 - Atrophic gastritis
 - Total gastrectomy
 - Blind loop syndrome
 - Chronic glossitis

Continued

REVIEW QUESTIONS—cont'd

9. Which of these statements is true regarding the testing of antibodies to intrinsic factor for diagnosing pernicious anemia?
 - a. Antibodies to IF are both sensitive and specific for the diagnosis of pernicious anemia.
 - b. Antibodies to IF are sensitive for the diagnosis of pernicious anemia but are not specific.
 - c. Antibodies to IF are specific for the diagnosis of pernicious anemia but are not sensitive.
 - d. Testing for antibodies to IF should not be part of the laboratory diagnosis for pernicious anemia.
10. Macrocytosis associated with acute blood loss is characterized by:
 - a. Decreased reticulocyte count
 - b. Increased reticulocyte count
 - c. Pancytopenia
 - d. Macroovalocytes
11. Which of the following laboratory results is associated with pernicious anemia and not macrocytic anemia due to liver disease?
 - a. Increased LD
 - b. Increased bilirubin
 - c. Increased MCV
 - d. Hypersegmented neutrophils
12. Which of the following laboratory findings coincides with megaloblastic anemia?
 - a. Increased serum iron and serum bilirubin
 - b. Decreased serum iron and serum bilirubin
 - c. Decreased serum muramidase
 - d. Increased haptoglobin
13. Which type of therapy is performed for vitamin B₁₂ deficiency?
 - a. Short-term vitamin therapy
 - b. Life-long vitamin therapy
 - c. Red blood cell transfusion
 - d. Intravenous injection
14. Which of the following lab results would differentiate a macrocytic anemia as nonmegaloblastic?
 - a. Macrocytes
 - b. Normal B₁₂ and folic acid levels
 - c. Macroovalocytes
 - d. Hypersegmented neutrophils

See answers at the back of this book.

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Hemolytic Anemias

Intracorpuscular Defects: Hereditary Defects of the Red Cell Membrane

Thérèse L. Coetzer, PhD

CHAPTER OUTLINE

Classification of Hemolytic Anemias

Approach to Diagnosis of a Hemolytic State

Tests Reflecting Increased Red Cell Destruction

Tests Reflecting Increased Red Cell Production

Establishing the Cause of Hemolysis

Hereditary Defects of the Red Cell Membrane

Red Cell Membrane Structure

Classification of Hereditary

Defects of the Red Cell

Membrane

Hereditary Spherocytosis

Hereditary Elliptocytosis

Disorders of Red Cell Hydration

Hereditary Hydrocytosis and

Hereditary Xerocytosis

Summary Chart

Case Study 9-1

Case Study 9-2

Case Study 9-3

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 9-1 Define intracorpuscular and extracorpuscular red blood cell defects relating to hemolytic anemia classification.
- 9-2 Contrast laboratory tests that reflect increased red cell destruction from those that reflect red cell production.
- 9-3 Calculate a reticulocyte production index.
- 9-4 Outline the approach and laboratory tests to classify the cause of red cell hemolysis.
- 9-5 Detail the structure of the red cell membrane.
- 9-6 Identify the molecular and red cell membrane defects associated with hereditary spherocytosis.

- 9-7 Recognize abnormal laboratory results associated with hereditary spherocytosis.
- 9-8 Name the functional abnormalities affecting membrane skeleton proteins in hereditary elliptocytosis and ovalocytosis.
- 9-9 Identify the abnormalities that cause the severe fragmentation and microspherocytosis characteristic of hereditary pyropoikilocytosis.
- 9-10 Recall laboratory findings associated with hereditary elliptocytosis disorders.
- 9-11 List disorders of red cell hydration and membrane cation permeability.

The red blood cell (RBC) membrane is critical for the function and survival of the cell. It is a key determinant of the unique biconcave disc shape, which provides flexibility and durability, allowing the RBC to withstand the circulatory shear forces and remain intact while negotiating the microvasculature and the spleen during its average 120-day life span.

Defects of the RBC membrane alter the shape and deformability of the cell, which leads to premature destruction and hemolysis. These disorders comprise an important group of hereditary hemolytic anemias and include hereditary spherocytosis (HS), hereditary elliptocytosis (HE) and related disorders, and disorders of RBC hydration. This chapter summarizes the laboratory approach to detect hemolysis, our current understanding of the underlying molecular defects, and the pathophysiology of these disorders.

Classification of Hemolytic Anemias

A hemolytic state exists when the *in vivo* survival of RBC is shortened. The presence of anemia in an individual patient is, however, dependent on the degree of hemolysis and the compensatory response of the erythroid elements of the bone marrow. Normal bone marrow is able to increase its output about six- to eightfold, so that anemia is not manifest until this capacity is exceeded, corresponding to an RBC life span of about 15 to 20 days or less. Anemia may, however, occur with more moderate shortening of the RBC life span if there is an associated depression of bone marrow function, which may occur with certain systemic diseases or exposure to chemicals or drugs.

A useful classification of the hemolytic anemias entails their subdivision into those disorders associated with an

intrinsic (intracorporeal) defect of the RBC and those associated with an **extrinsic (extracorporeal) abnormality**. RBCs from a patient with an intracorporeal defect have a shortened survival in both the patient and a normal recipient, whereas normal donor RBCs survive normally in the patient. In contrast, normal RBCs are destroyed more rapidly when transfused into a patient with an extracorporeal abnormality. The patient's RBCs, when transfused into a healthy recipient, have normal survival, provided that they have not been irreversibly damaged. Hemolytic states have also traditionally been regarded as **intravascular** or **extravascular**; that is, sequestration occurs in reticuloendothelial tissue. However, vigorous extravascular hemolysis may often be associated with signs of hemoglobin release into the plasma such as hemoglobinemia and decreased haptoglobin levels. The distinction is still useful from a clinical standpoint because certain hemolytic states are associated with predominantly intravascular hemolysis (e.g., paroxysmal nocturnal hemoglobinuria and infections caused by *Clostridium* or *Plasmodium falciparum*).

Hemolytic anemias may be classified as outlined in Table 9-1.

Approach to Diagnosis of a Hemolytic State

The approach to the diagnosis of hemolytic anemia initially involves establishing an increased rate of RBC destruction and then focuses on determining the cause of hemolysis.

Diagnostic tests to establish the presence of hemolysis rely on the fact that hemolysis is characterized by both increased RBC destruction and increased production.

Tests Reflecting Increased Red Cell Destruction

Intravascular or extravascular destruction of RBCs releases hemoglobin, which is processed and degraded in a series of steps, as depicted in Figure 9-1. Several tests detect catabolic intermediates in this cascade, and the two most frequently used are the serum **unconjugated (indirect) bilirubin** and serum **haptoglobin** determinations. RBCs phagocytosed by the reticuloendothelial system result in the release of **bilirubin**, a breakdown product of heme, which elevates the level of unconjugated bilirubin in the serum, although it seldom exceeds 3 to 4 mg/dL in uncomplicated hemolytic states. A decreased serum haptoglobin level is a sensitive measure of

both intravascular and extravascular hemolysis, and it reflects the rapid clearance of a complex formed between liberated hemoglobin and circulatory haptoglobin. Drawbacks to the use of serum haptoglobin levels are that low levels may occur in hepatocellular disease, reflecting decreased synthesis by the liver, and that some individuals, particularly in black African populations, may have a genetically determined deficiency of haptoglobin. Increased synthesis of haptoglobin in acute inflammatory states or malignancy may also mask depletion of serum haptoglobin caused by hemolysis.

In addition to the two tests described, measurement of **lactate dehydrogenase (LDH)** is typically part of a standard workup for hemolysis. LDH is an enzyme present in RBCs, and hemolysis causes release into the plasma, which elevates the level. This is a sensitive test but not specific for hemolysis since LDH is ubiquitous and can be released from other damaged cells.

Urinalysis may be useful to reveal increased RBC destruction, particularly if it is primarily intravascular, by detecting hemoglobinuria and hemosiderinuria (Fig. 9-1). Once the hemoglobin-binding capacity of serum haptoglobin is exceeded, hemoglobin passes into the kidney, where it is reabsorbed and degraded. The liberated iron is conserved as ferritin and hemosiderin, which can be detected in the urine several days after a hemolytic episode. When the tubular reabsorptive capacity for hemoglobin is exceeded, hemoglobinuria ensues.

Tests Reflecting Increased Red Cell Production

The compensatory bone marrow response to hemolysis results in the delivery of young RBCs in the form of **reticulocytes** into the circulation. These young cells contain RNA, which stains supravitality with dyes such as new methylene blue or brilliant cresyl blue. The normal reticulocyte count has a range of 0.5% to 2.0%, reflecting the fact that, each day, approximately 1% of the RBC mass is destroyed and replaced by young RBCs from the bone marrow, because RBC survival is approximately 120 days. The reticulocyte count is always elevated in a hemolytic state in which there is a normal compensatory bone marrow response. However, a more accurate assessment of RBC production is required, because the percentage of reticulocytes may be "spuriously" elevated as the reticulocytes may be diluted into a lesser number of total circulating RBCs.

TABLE 9-1 Classification of Hemolytic Anemias

Type of Defect	Hereditary	Acquired
Intracorporeal	<ul style="list-style-type: none"> Defects in the red cell membrane Enzyme defects Hemoglobinopathies Thalassemia syndromes 	Paroxysmal nocturnal hemoglobinuria
Extracorporeal		<ul style="list-style-type: none"> Immune hemolytic anemias Infections Exposure to chemicals and toxins Exposure to physical agents Microangiopathic and macroangiopathic hemolytic anemias Splenic sequestration (hypersplenism) General systemic disorders (in which hemolysis is not the dominant feature of the anemia)

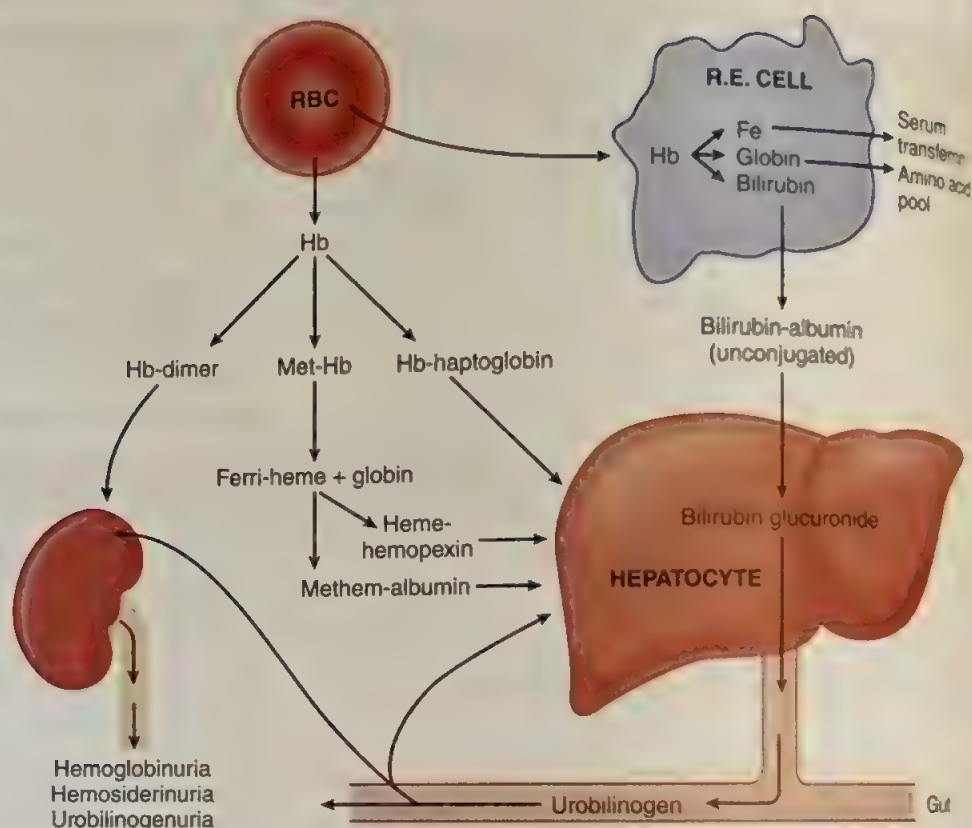


FIGURE 9-1 Diagrammatic representation of the degradation of hemoglobin after intravascular or extravascular destruction of red cells. Fe = iron; Hb = hemoglobin; RBC = red blood cell; R.E. = reticuloendothelial cell.

In addition, in response to the anemia, reticulocytes may leave the bone marrow prematurely and mature in the circulation for longer than the normal maturation time of 1 day, again leading to a falsely elevated reticulocyte count. These cells (so-called shift reticulocytes) are recognizable as large bluish-gray erythrocytes on Romanowsky (Wright, Giemsa) stains.

The **reticulocyte production index (RPI)** corrects the hematocrit to a normal value of 45% and considers the maturation time of the reticulocyte at a particular hematocrit (approximately 1.0 day at a hematocrit of 45%, 1.5 days at 35%, 2.0 days at 25%, and 2.5 days at 15%).¹

$$\text{RPI} = \frac{\% \text{ Reticulocytes}}{\text{Reticulocyte maturation time}} \times \frac{\text{Hematocrit \%}}{45\%}$$

For example, an RPI of 5.3 is calculated for a patient suspected of having a hemolytic state with the following indices: hemoglobin, 12.0 g/dL; hematocrit, 36%; reticulocyte count, 10%; shift cells present.

An RPI of greater than 2.5 to 3.0 is generally regarded as indicative of hemolysis, but it is very important to exclude the presence of hemorrhage, as this too may lead to an elevated RPI. Although the RPI is probably the single most useful test to detect a hemolytic state, a cautionary note is in order, as the test may not be sensitive enough to detect mild cases.

Establishing the Cause of Hemolysis

Once having documented the presence of hemolysis, the approach followed by Lux and Glader² in establishing the cause of hemolysis is pragmatic and logical, and this is the technique followed in this chapter and shown diagrammatically in Figure 9-2. The initial step consists of separating patients into Coombs' test-positive (i.e., immunohemolytic anemias) and Coombs' test-negative groups. The latter group is then

further divided into "smear-positive" and "smear-negative nonspecific" subgroups. It is fundamentally important to assess morphology in peripheral smears that are free of artifact. On the basis of the classification according to the predominant morphology associated with a particular disease state (Box 9-1), it is possible to considerably narrow the differential diagnosis and then institute further appropriate tests to make a definitive diagnosis.

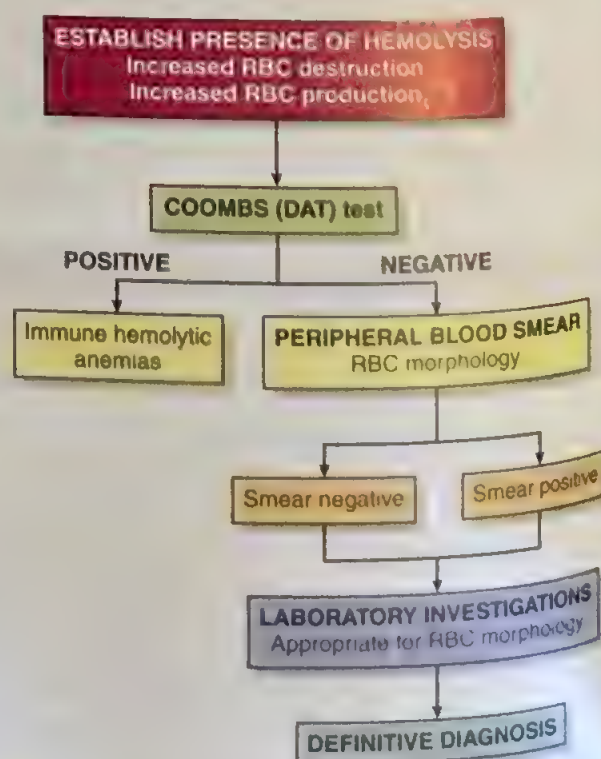


FIGURE 9-2 Diagnostic approach to hemolytic anemias.

BOX 9-1 Predominant Red Cell Morphology Commonly Associated With Nonimmune Hemolytic Disorders**Spherocytes**

- Hereditary spherocytosis
- Acute oxidant injury (HMP shunt defects during hemolytic crisis, oxidant drugs and chemicals)
- *Clostridium welchii* septicemia
- Severe burns, other red cell thermal injuries
- Spider, bee, and snake venoms
- Severe hypophosphatemia

Elliptocytes

- Hereditary elliptocytosis
- Thalassemias
- Iron deficiency
- Megaloblastic anemia

Bizarre Poikilocytes

- Red cell fragmentation syndrome (microangiopathic and macroangiopathic hemolytic anemias)
- Hereditary elliptocytosis in neonates
- Hereditary pyropoikilocytosis

Stomatocytes

- Hereditary stomatocytosis and related disorders
- Stomatocytic elliptocytosis

Irreversibly Sickled Cells

- Sickle cell anemia
- Symptomatic sickle syndromes

Intraerythrocytic Parasites

- Malaria
- Babesiosis
- Bartonellosis

Prominent Basophilic Stippling

- Thalassemias
- Unstable hemoglobins
- Lead poisoning
- Pyrimidine-5'-nucleotidase deficiency

Spiculated or Crenated Red Cells

- Acute hepatic necrosis (spur cell anemia)
- Uremia
- Infantile pyknocytosis
- Abetalipoproteinemia
- McLeod blood group

Target Cells

- Hemoglobins S, C, D, and E
- Thalassemias
- Hereditary xerocytosis

Nonspecific or Normal Morphology

- Embden-Meyerhof pathway defects
- HMP shunt defects
- Adenosine deaminase hyperactivity with low red cell ATP
- Unstable hemoglobins
- Paroxysmal nocturnal hemoglobinuria
- Dyserythropoietic anemias
- Copper toxicity (Wilson's disease)
- Cation permeability defects
- Erythropoietic porphyria
- Vitamin E deficiency
- Hypersplenism

ATP = adenosine triphosphate; HMP = hexose monophosphate.

It is also worth emphasizing that many hemolytic states are associated with an underlying disease, as will become apparent in the ensuing chapters, and this should be considered in the assessment of the individual patient.

CRITICAL THINKING QUESTION

9-1 Which steps are required to establish a diagnosis of hemolytic anemia?

See answers to all Critical Thinking Questions at the back of this book.

Hereditary Defects of the Red Cell Membrane**Red Cell Membrane Structure**

An understanding of the etiology and pathophysiology of hemolytic states caused by defects of the RBC membrane requires some knowledge of its structural organization. The membrane consists of a relatively fluid **phospholipid bilayer** stabilized by interactions with integral membrane proteins within the bilayer and with the underlying peripheral membrane protein skeleton. The membrane provides the RBC with the necessary strength

and flexibility to survive the circulatory shear stress and numerous passages through the spleen during its 4-month life span. The ability of the red cell to deform and subsequently regain its original biconcave disc shape is determined by three factors:

1. Cell surface area-to-volume ratio
2. The viscoelastic properties of the membrane, which depend on the structural and functional integrity of the membrane skeleton
3. The cytoplasmic viscosity, which is determined primarily by intracellular hemoglobin

The structural organization of the protein and lipid components of the RBC membrane has been reviewed in Chapter 2, and only some aspects of the membrane proteins implicated in the pathogenesis of hemolytic anemia are emphasized here.

The **RBC membrane skeleton** underlies the lipid bilayer and is a loosely knit two-dimensional protein network consisting mainly of the structural proteins α - and β -spectrin, actin, protein 4.1R, and actin-associated proteins.³ Negatively stained stretched skeletons viewed via high-resolution electron microscopy⁴ reveal a hexagonal lattice of predominantly spectrin tetramers with some hexamers joined together by junctional protein complexes (Fig. 9-3). These junctional complexes are mainly composed of protein 4.1R, short F-actin

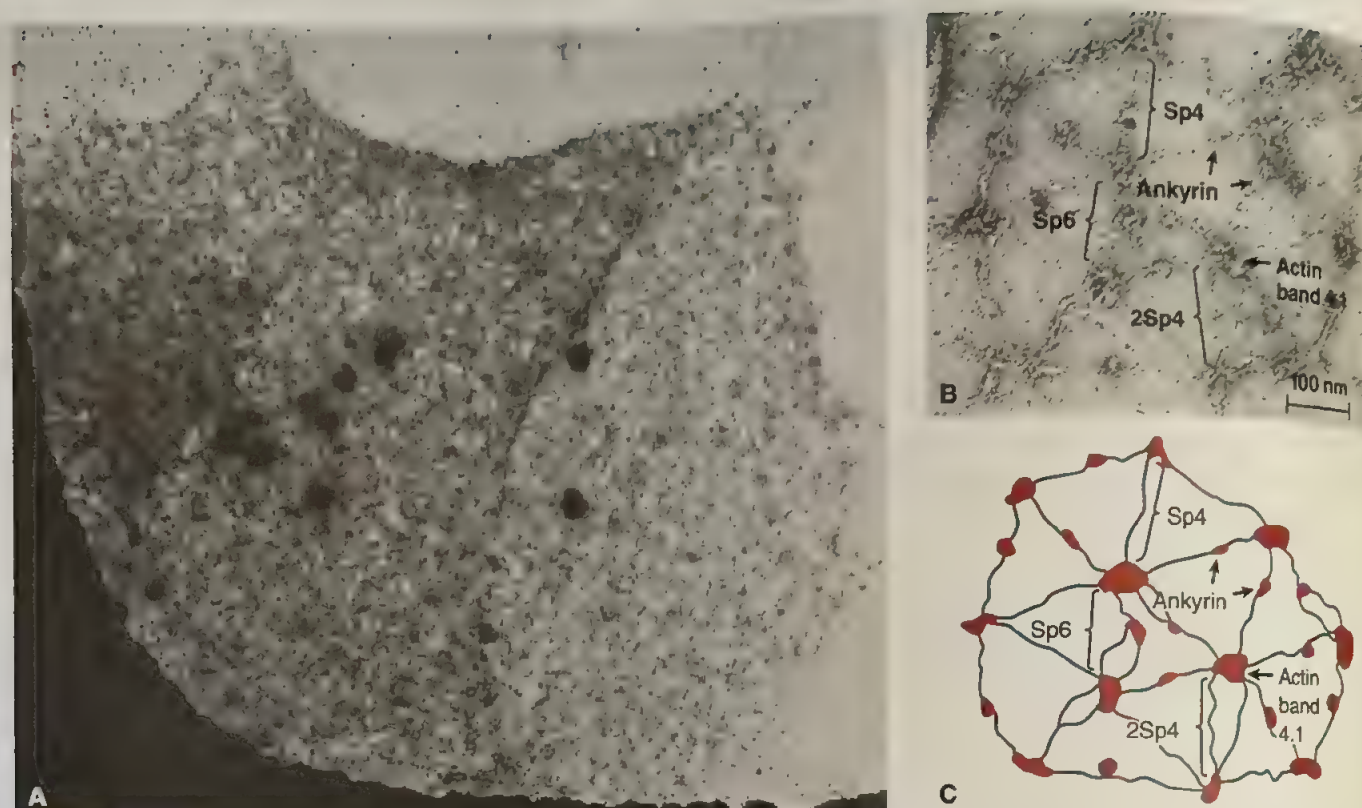


FIGURE 9-3 Transmission electron micrographs of negatively stained red cell membrane skeletons. **A**, An area of spread skeleton network. **B, C**, The hexagonal lattice made up of spectrin tetramers (Sp4), hexamers (Sp6), or double tetramers (2Sp4). Cross-linking junctional complexes contain short F-actin filaments and protein 4.1R (band 4.1). Globular ankyrin structures are bound to spectrin filaments about 80 nm from their distal ends. (From Liu, SC, et al. Visualization of the hexagonal lattice in the erythrocyte membrane skeleton. *J Cell Biol.* 1987;104:527, with permission.)

filaments, and actin-associated proteins including adducin, dematin (protein 4.9), protein p55, tropomyosin, and tropomodulin.⁵ The spectrin/actin skeleton is anchored to the phospholipid bilayer by interactions with integral membrane proteins forming multiprotein complexes.^{3,5} The primary anchoring sites are ankyrin complexes based on a high-affinity interaction of ankyrin with both the transmembrane anion exchanger-1 (AE1) and β -spectrin. Several other integral and peripheral proteins are involved, including protein 4.2. A

secondary attachment is mediated by the junctional complex whereby protein 4.1R interacts with AE1 and glycophorin C. Spectrin and protein 4.1R also interact directly with the phospholipid bilayer.

Red cell **spectrin**, the major skeletal protein,³ is an elongated flexible heterodimer composed of stoichiometric amounts of two structurally related, but functionally distinct, α and β polypeptides that are encoded by separate genes, *SPTA1* and *SPTB*, respectively (Table 9-2).

TABLE 9-2 Properties of Selected Red Cell Membrane Proteins Implicated in Hemolytic Anemia

Protein	SDS-PAGE Band*	Molecular Mass [†] (kD)	Gene Symbol	Chromosomal Localization	Diseases
Skeletal Proteins					
α -Spectrin	1	280	<i>SPTA1</i>	1q21 → q23	HS, HE, HPP
β -Spectrin	2	246	<i>SPTB</i>	14q23 → q24.1	HS, HE, HPP
Protein 4.1R	4.1	66	<i>EPB41</i>	1p33 → p34.2	HE
Integral Proteins					
AE1 (Band 3)	3	102	<i>SLC4A1</i>	17q21.31	HS, SAO
Glycophorin C	PAS-2	14	<i>GYPC</i>	2q14 → q21	HE
Linker Proteins					
Ankyrin	2.1	206	<i>ANK1</i>	8p11.2	HS
Protein 4.2	4.2	77	<i>EPB42</i>	15q15 → q21	HS

*Proteins were separated by SDS-PAGE and stained with Coomassie blue or periodic acid-Schiff reagent (PAS).

[†]Molecular weight in kilodaltons (kD) is calculated from the amino acid sequence.

HS = hereditary spherocytosis; HE = hereditary elliptocytosis; HPP = hereditary pyropoikilocytosis; SAO = Southeast Asian ovalocytosis.

ADVANCED CONTENT

Both α - and β -spectrin can be subdivided into structural domains (α I- α V and β I- β IV) that are resistant to mild proteolysis by trypsin (Fig. 9-4A). Each spectrin molecule consists of several homologous 106-amino-acid repeats that fold into coiled antiparallel triple helices,⁶ which render the molecule highly flexible and enable the RBC to survive the circulatory shear stress. The C-terminal of the 280 kD α -spectrin has an EF hand motif, which binds calcium. The N-terminal of the 246 kD β -spectrin contains binding sites for protein 4.1R, actin, and adducin, and repeats 14-15 form a binding pocket for ankyrin (Fig. 9-4A). The α and β monomers interact along their length in an antiparallel fashion starting at nucleation sites at the tail end of the molecules. The head regions of the $\alpha\beta$ heterodimers self-associate into tetramers via an interaction between the two helices from partial repeat 17 of the β I domain at the phosphorylated C-terminal, and a third helix from the N-terminal of the α I domain to form a complete triple helix. An important aspect in the biogenesis of the membrane skeleton is that α -spectrin is synthesized in approximately threefold excess over β -spectrin and undergoes slower degradation, indicating that β -spectrin is the rate-limiting component in spectrin assembly.⁷

The major integral membrane protein is AE1, previously known as band 3, encoded by the *SLC4A1* gene (Table 9-2).⁸ As an anion exchanger, it performs an important transport function by regulating $\text{HCO}_3^-/\text{Cl}^-$ exchange and facilitating the transfer of carbon dioxide from tissues to lungs. The 102 kD protein is divided into two structurally and functionally distinct domains (Fig. 9-4B). The 43 kD N-terminal cytoplasmic domain contains binding sites for ankyrin and proteins 4.1 and 4.2, and also binds glycolytic enzymes and hemoglobin. The 52 kD C-terminal region has 14 transmembrane segments intercalated in the lipid bilayer that form the anion-exchange channel.

Ankyrin is the major linker protein that connects the membrane skeleton to the bilayer, and it is encoded by the *ANK1* gene (Table 9-2).^{3,5,9} The 206 kD protein has an N-terminal 89 kD domain, consisting of 24 tandem ANK repeats containing 33 amino acids each (Fig. 9-4C), which bind to AE1. Spectrin binds to the central 62 kD domain of ankyrin, whereas the 55 kD C-terminal portion is a regulatory domain that is alternatively spliced at the mRNA level, yielding ankyrin isoforms, which modulate the interaction with spectrin and AE1.

Protein 4.1R is a 66 kD globular protein with four structural domains as depicted in Figure 9-4D.^{3,5} A 10-kD domain enhances the spectrin-actin interaction by binding

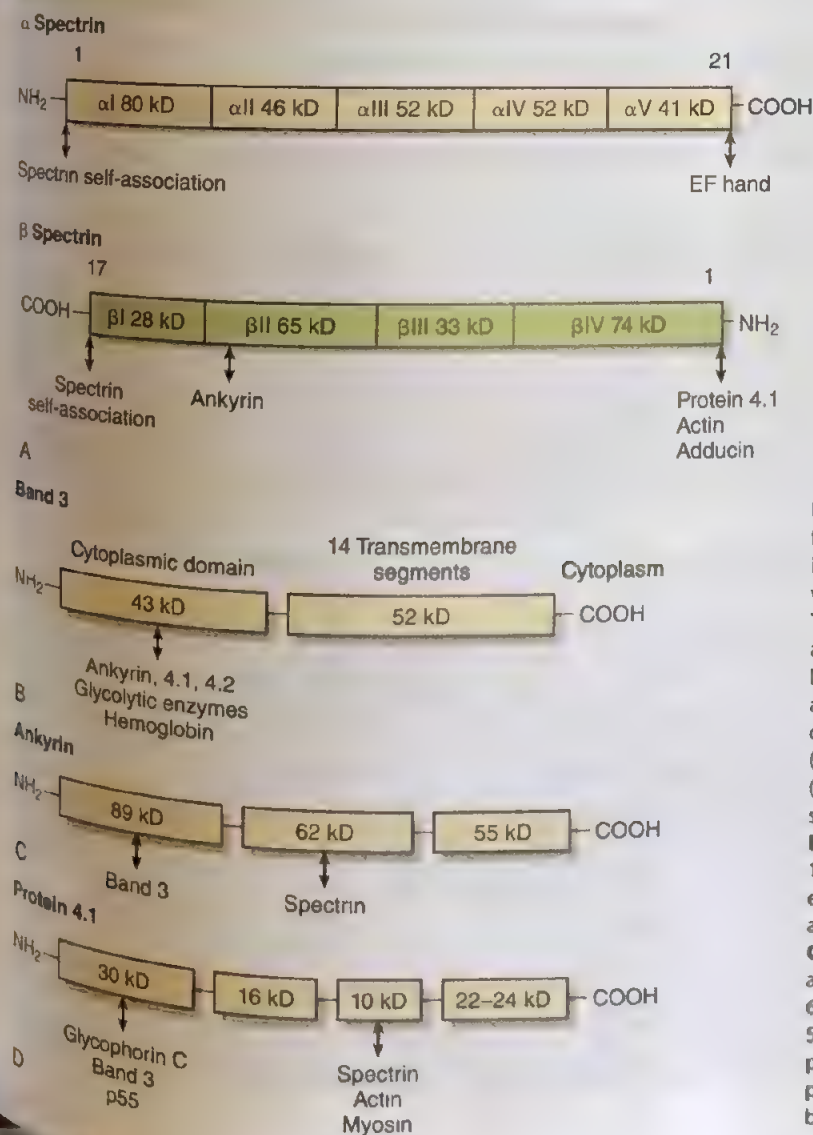


FIGURE 9-4 Schematic representation of the structure of four of the red cell membrane proteins involved in hereditary hemolytic anemias. The molecular masses of the various domains are given in kilodaltons (kD). **A.** Spectrin. The subunit structure of α - and β -spectrin, showing the antiparallel arrangement of the N- and C-terminals, the homologous triple helical spectrin repeat units (indicated above the α and β chains), and the trypsin-resistant domains (open squares). α -Spectrin has five domains (α I to α V) and 21 repeats. β -Spectrin has four domains (β I to β IV) and 17 repeats. Proteins interacting with spectrin are indicated below the relevant domains. **B.** Band 3 (AE1). The 52-kD membrane domain contains 14 transmembrane segments and is responsible for anion exchange. The 43-kD N-terminal cytoplasmic domain and the proteins binding to this domain are shown. **C.** Ankyrin. The N-terminal 89-kD domain consists of 24 ankyrin repeat units, which bind to band 3 (AE1). The 62-kD domain interacts with spectrin and the C-terminal 55-kD regulatory domain modulates the activity of the protein. **D.** Protein 4.1. The four structural domains of the protein are depicted as open rectangles and the proteins binding to each domain are shown below.

to both proteins, as well as to myosin, and the N-terminal 30 kD domain interacts with glycophorin C, p55, and AE1. The *EPB41* gene produces mRNA that is subject to extensive alternative splicing, yielding tissue- and development-specific isoforms. Post-translational deamidation of asparagine 502 occurs during development of reticulocytes into mature red cells, and these two forms are designated protein 4.1Ra and b.

The genes coding for the major membrane proteins have been sequenced and their chromosomal localization identified (Table 9-2). Mutations in any of these genes that alter the amount or function of the expressed proteins compromise the integrity of the membrane and manifest in altered RBC morphology. These cells are unable to survive passage through the spleen, resulting in premature destruction and hemolytic anemia.

Classification of Hereditary Defects of the Red Cell Membrane

The hereditary hemolytic anemias due to red cell membrane protein defects may be classified according to the morphological abnormality of the red cells. Four main groups are delineated:

1. Hereditary spherocytosis (HS)
2. Hereditary elliptocytosis (HE) and morphologically related disorders, including hereditary pyropoikilocytosis (HPP) and Southeast Asian ovalocytosis (SAO)
3. Hereditary hydrocytosis (stomatocytosis)
4. Hereditary xerocytosis

By far the most common and well-characterized groups of disorders are HS and HE, and this chapter focuses on these important entities. In recent years, there have been major advances in our understanding of the molecular basis of these disorders. To

gain insight into the pathogenesis and to enable a correlation of the genotype with the observed morphological phenotype, it is useful to divide the interactions between the red cell membrane components into two categories (Fig. 9-5), as follows

1. **Vertical interactions** occur between the membrane skeleton and the bilayer and mainly involve spectrin-ankyrin-AE1 associations, as well as weak contacts between spectrin and the negatively charged lipids of the inner half of the membrane bilayer.
2. **Horizontal interactions** occur between components of the membrane skeleton and include spectrin dimer self-association to produce tetramers and spectrin-actin-protein 4.1R complex formation.

As first predicted by Jiri Palek in 1984¹⁰ and subsequently verified by mutation analyses and other studies, defects in vertical interactions manifest as spherocytosis, whereas defects in horizontal interactions lead to elliptocytosis (Fig. 9-5).

CRITICAL THINKING QUESTION

9-2 How do the proteins that are affected by genetic mutation cause hereditary defects of the red blood cell membrane?

Hereditary Spherocytosis

Inheritance and Epidemiology

HS is characterized by osmotically fragile, spherical red cells and is the most common hereditary hemolytic anemia in people of Northern European origin (Fig. 9-6).^{11,12} It has been

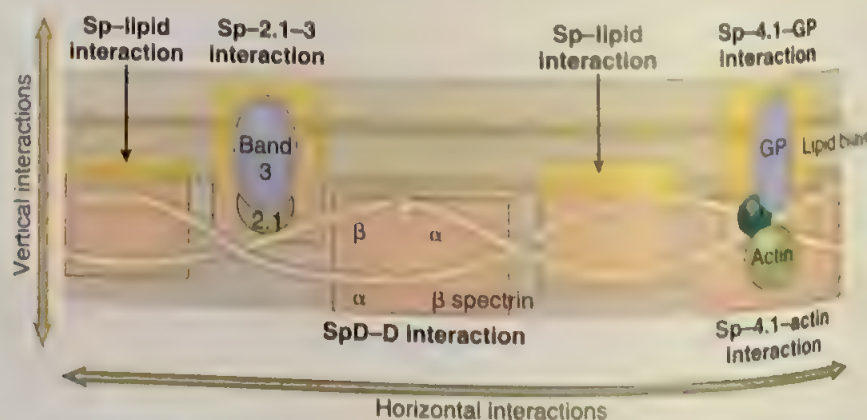
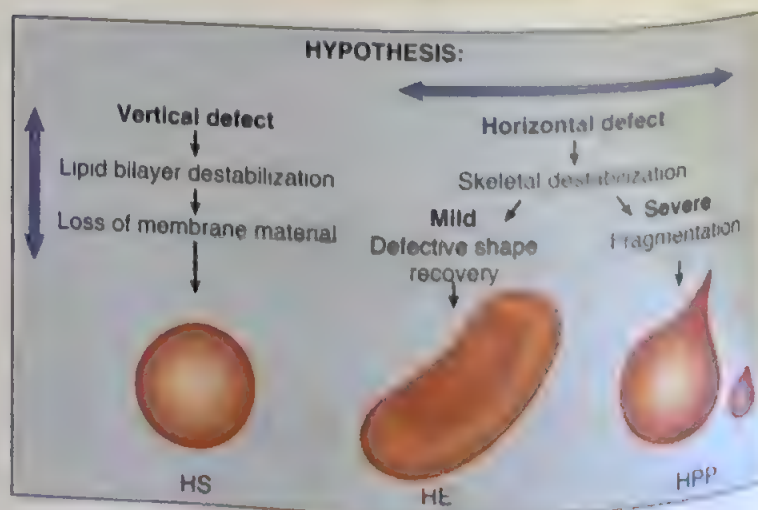


FIGURE 9-5 Diagrammatic illustration of the vertical and horizontal interactions between the red cell membrane components (top). The bottom section illustrates the pathophysiology of the red cell lesion in hereditary spherocytosis (HS), hereditary elliptocytosis (HE), and hereditary pyropoikilocytosis (HPP). A defect in a vertical interaction resulting in spherocytes and HS is illustrated at the bottom left. A defect in a horizontal interaction resulting in elliptocytes and poikilocytes (HE and HPP) is illustrated at the bottom right. (From Palek, J. Disorders of red cell membrane skeleton: An overview. In Kruckeberg, WL, et al (Eds). Erythrocyte Membranes 3: Recent Clinical and Experimental Advances. New York: Alan R. Liss; 1984, p. 177, with permission.)



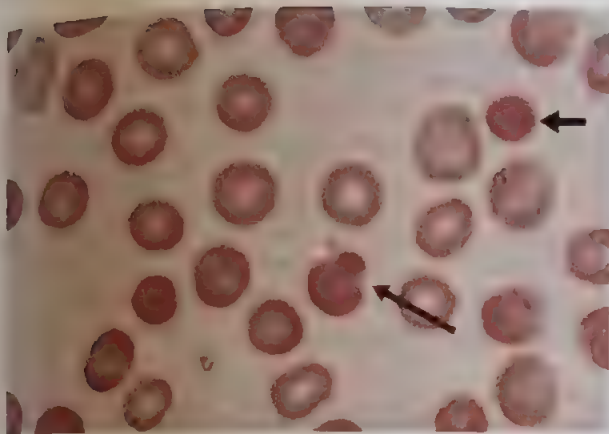


FIGURE 9-6 Photomicrograph of peripheral blood smear from a patient with hereditary spherocytosis (HS). Note the microspherocytes (small condensed spherocytes with no central pallor), indicated by the small black arrow and the pincered (mushroom-shaped) cell, indicated by the long black arrow.

documented in all race groups, including Japanese and black Africans from Southern Africa. In at least 75% of cases, it follows a classic autosomal dominant pattern of inheritance, but in the remaining families both parents are clinically normal, suggesting autosomal recessive inheritance or variable penetrance of a dominant gene or a *de novo* mutation.

Molecular Defects

The underlying molecular defects in HS are heterogeneous, and several genetic loci have been implicated.^{11,12,13} In the vast majority of cases, the resulting protein abnormalities are quantitative with decreased amounts of spectrin, ankyrin, AE1, or protein 4.2 (Box 9-2). These defects involve vertical interactions between the bilayer and the skeleton and are found worldwide, but the relative frequency of each abnormality varies between countries and ethnic groups. More than 200 different causative mutations have been identified and are generally unique to each kindred.

Spectrin deficiency is a common underlying cause of HS, and it was first documented by Agre and coworkers,¹⁴ who noted that the number of spherocytes, severity of the disease, and response to splenectomy correlated closely with the reduced spectrin content. Spectrin deficiency may be secondary to a decreased amount of ankyrin because the loss of ankyrin attachment sites prevents binding of spectrin to AE1. Mutations in the *SPTA1* gene have no effect in the heterozygous state, because α -spectrin is synthesized in excess. However, biallelic *SPTA1* mutations have been implicated

in severe recessive HS.^{13,15} β -Spectrin defects are associated with dominant HS because β -spectrin synthesis is rate limiting in the assembly of spectrin in the membrane. Several different mutations in the *SPTB* gene have been described in individual families.^{11,12,13,16} These include missense mutations, splice-site mutations, as well as insertions or deletions, which ultimately result in a shift in the reading frame of the *SPTB* gene, creating abnormal stop codons that terminate translation prematurely. These, together with nonsense mutations, as well as point mutations in the translation initiation codon that prevent translation of the protein, silence the expression of the mutant allele and manifest as null mutations.

Ankyrin deficiency, resulting in a concomitant decrease in spectrin, was first described by Coetzer and coworkers,¹⁷ and subsequently shown to be caused by decreased ankyrin mRNA production and synthesis of an unstable molecule. Mutations in the *ANK1* gene are common,^{11,12,16} especially in HS patients from Europe and North America. Interstitial deletions of chromosome 8 involving the *ANK1* gene (8p11.2), as well as balanced translocations involving chromosome 8p11, have been reported.^{11,12} Nonsense and frameshift mutations, which result in either unstable mRNA transcripts or truncated proteins with functional abnormalities or decreased stability, are common, but missense and promoter mutations have also been documented.^{11,12,13,16}

AE1 deficiency often results in a secondary deficiency of protein 4.2 due to the reduction in protein 4.2 binding sites in the cytoplasmic domain of AE1. Null mutations are common and may be caused by single nucleotide insertions or deletions that alter the reading frame of the mutant *SLC4A1* allele or by nonsense mutations or splicing defects.^{11,12,13} Small in-frame deletions or insertions have been documented in a few cases. *SLC4A1* missense mutations are very common, and these often result in substitution of highly conserved amino acids, such as arginine residues at the internal boundaries of the transmembrane segments that play a crucial role in the stabilization of the AE1 protein and its insertion into the membrane.¹⁸ Several of these arginine residues (encoded by CGN) are mutation "hotspots." The abnormal amino acids may also influence the anion transport function of AE1 if they are located in the transmembrane domain or alter the binding of protein 4.2 if they occur in the cytoplasmic domain. Homozygous AE1 null mutations have been described in HS patients who are severely affected, indicating that long-term survival without the AE1 protein is possible.¹⁹

Protein 4.2 deficiency is rare in Europeans but relatively common in Japanese patients with recessively inherited HS who exhibit almost complete absence of the protein.^{11,12} A small number of recurrent causative mutations in the *EBP42* gene have been described, including a missense mutation resulting in protein 4.2 Nippon.²⁰

Pathophysiology

The fundamental expression of the membrane defect in HS is a loss of surface area of the red cell, resulting in a decreased surface-to-volume ratio. This is manifested morphologically as spherocytosis, although it should be noted that the majority of HS cells are spherostomatocytic rather than truly spherocytic.

BOX 9-2 Defects of Red Cell Membrane Proteins in Hereditary Spherocytosis

Protein Deficiencies

- Spectrin
- Ankyrin
- Anion exchanger (AE1, Band 3)
- Protein 4.2

earlier, but in either event, treatment with pneumococcal vaccine is recommended, preferably starting before splenectomy. Younger children may also require prophylactic penicillin or other antibiotics postsplenectomy, but the latter course is controversial. Failure of splenectomy is almost always associated with an accessory spleen not removed at surgery or more rarely is caused by autotransplantation of splenic tissue in the peritoneal cavity, leading to splenosis. Laparoscopic splenectomy and partial splenectomy are options that may be considered.^{11,12}

CRITICAL THINKING QUESTION

9-3 Explain the classic triad presentation seen in hereditary spherocytosis.

Hereditary Elliptocytosis

Inheritance and Epidemiology

Hereditary elliptocytosis (HE) is a heterogeneous group of disorders found worldwide in all race groups and characterized by the presence of elliptical red cells in the peripheral blood (Fig. 9-8). HE syndromes are usually inherited in an autosomal dominant fashion, except for hereditary pyropoikilocytosis (HPP), which is a recessive and very severe rare disorder. Common HE is the most prevalent condition. Southeast Asian ovalocytosis (SAO) is very common in Melanesian and other Southeast Asian population groups because of the protective effect toward malaria. SAO exhibits autosomal dominant inheritance, and homozygosity is lethal in utero without intervention.

Molecular Defects

Hereditary Elliptocytosis The underlying abnormality in HE resides in the red cell membrane skeleton and usually involves spectrin or protein 4.1 (Box 9-3; Table 9-3).

Spectrin mutations that impair the self-association of spectrin dimers into tetramers are the most common. This functional defect is caused by an alteration in the structure of the spectrin self-association site, usually involving the N-terminal

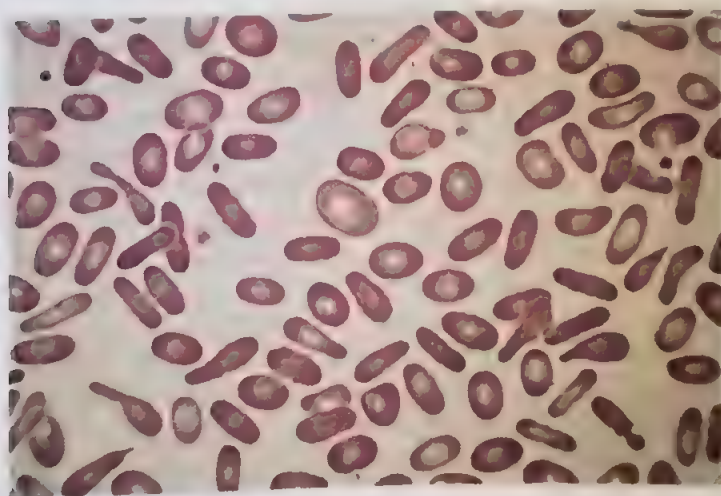


FIGURE 9-8 Photomicrograph of peripheral blood smear from a patient with mild hereditary elliptocytosis (HE). Note the high percentage of elliptocytes.

BOX 9-3 Defects of Red Cell Membrane Proteins in Hereditary Elliptocytosis

Protein Dysfunction

- Spectrin dimer self-association
 - α -Spectrin mutations
 - β -Spectrin mutations
- Truncated α - or β -spectrin
- Spectrin-ankyrin interaction
- Protein 4.1R-spectrin interaction
- Anion exchanger (AE1, Band 3) (SAO)

Protein Deficiencies

- Protein 4.1
- Glycophorin C
- Spectrin (HPP)

HPP = hereditary pyropoikilocytosis; SAO = Southeast Asian ovalocytosis.

α I domain of α -spectrin, or less frequently, the C-terminal domain of β -spectrin. This disrupts the 106-amino acid helical spectrin repeats and alters the partial tryptic cleavage pattern of spectrin. The affected domain, as well as the size of the abnormal tryptic peptide, is used in the nomenclature of these defects, and Sp α I/74, Sp α I/65, and Sp α I/46 are the most common. The extent of impairment of spectrin dimer self-association and the amount and type of abnormal tryptic peptide correlate with the clinical severity, with Sp α I/74 being the most severe.²³ More than 70 causative mutations have been described in both α - and β -spectrin genes, and the most common are missense point mutations that substitute highly conserved amino acids.

ADVANCED CONTENT

Sp α I/74 mutations are close to the **dimer self-association** site and mainly cluster in exon 2 of the *SPTA1* gene. Codon 28, CGT, is a "hotspot" for mutations, whereby the normal arginine is replaced by one of four different amino acids.²⁴ In-frame deletions and splice site mutations have also been reported. Sp α I/46 mutations are distal from the self-association site and often involve the substitution of an amino acid with a proline residue, which is a helix breaker. Sp α I/65 is invariably caused by a duplication of codon 154, which inserts an additional leucine into the protein. This is a very mild defect and is common in Central and North Africa.²⁵

Mutations in the *SPTB* gene include missense point mutations, as well as frameshift errors caused by small deletions, insertions, or splice-site mutations that result in mutant β -spectrin molecules with truncated C-terminal domains.

A decreased amount of protein 4.1R has also been implicated in HE.¹¹ This is usually the result of mutations influencing the translation initiation site or, more rarely, deletions that shorten the protein or abolish spectrin binding.

TABLE 9-3 Characteristics of Hereditary Elliptocytosis Phenotypes

Phenotype	Hemolysis	Morphology	Most Common Defects
Common HE	Asymptomatic to mild	Elliptocytes	Impaired spectrin tetramer formation or Protein 4.1R deficiency
HE with infantile poikilocytosis	Moderately severe up to age 2 years	Elliptocytes Poikilocytes	Impaired spectrin tetramer formation or Protein 4.1R deficiency
HPP	Severe	Microspherocytes Poikilocytes Few elliptocytes	Severely impaired spectrin tetramer formation and spectrin deficiency
SAO	Asymptomatic	Large ovalocytes	9-amino acid deletion in AE1

HE = hereditary elliptocytosis; HPP = hereditary pyropoikilocytosis; SAO = Southeast Asian ovalocytosis.

Hereditary Pyropoikilocytosis HPP is an interesting, relatively rare, severe hemolytic disease that is part of the HE group of disorders. The peripheral blood smear is characterized by microspherocytosis, micropoikilocytosis, fragments, and relatively few, if any, elliptocytes (Fig. 9-9). Patients with HPP are compound heterozygotes, and all cases thus far investigated exhibit two genetic defects:

1. A mutant α - or β -spectrin that shows severe impairment of spectrin dimer self-association.²³
2. A partial spectrin deficiency resulting from decreased synthesis of α -spectrin, which causes the characteristic microspherocytosis.²⁶

Southeast Asian Ovalocytosis SAO is a very common, asymptomatic condition in ethnic Southeast Asian populations. SAO is characterized by rigid, spoon-shaped ovalocytes (Fig. 9-10) that are resistant to invasion by malaria parasites and provide an example of the selective pressure of malaria.²⁷ The underlying molecular defect is an in-frame deletion of 27 base pairs in exon 11 of the *SLC4A1* gene, resulting in the absence of nine amino acids at the junction of the cytoplasmic and transmembrane domains of the AE1

protein.²⁸ This alters the structure and function of the mutant AE1, causing increased binding to ankyrin, an inability to transport anions, and markedly restricted lateral and rotational mobility in the membrane.²⁹

Pathophysiology of Elliptocytosis and Pyropoikilocytosis

The molecular defects in HE and HPP involve horizontal interactions between proteins of the membrane skeleton (Fig. 9-5). Defective spectrin dimer self-association compromises the formation of spectrin tetramers, and protein 4.1R deficiency decreases the linkage of spectrin tetramers at the junctional complex. These abnormalities weaken the skeleton, and under the influence of shear stress in the circulation, the cells become distorted and progressively lose their ability to regain their original biconcave disc shape, resulting in elliptocytes and poikilocytes. The clinical expression of HE is variable and can differ between individuals of the same family with the same mutant allele. A low-expression α -spectrin allele, SpLELY, is an example of a modifying polymorphism that exacerbates the clinical phenotype when inherited in *trans* to a mutant α -spectrin allele.^{11,30}



FIGURE 9-9 Photomicrograph of peripheral blood smear from a patient with hereditary pyropoikilocytosis (HPP). Note the bizarre micropoikilocytosis, red cell budding, and very few elliptocytes.

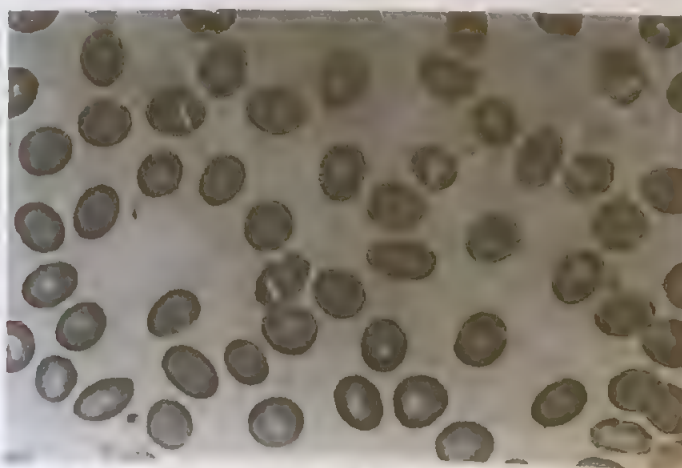


FIGURE 9-10 Photomicrograph of peripheral blood smear from a patient with Southeast Asian ovalocytosis (SAO). Note the characteristic spoon-shaped oval cells with a band across the central area.

In the case of HPP, the clinical severity and morphology are thought to result from a combination of horizontal and vertical defects. Spectrin self-association (horizontal defect) is severely impaired in HPP, which markedly decreases the strength and stability of the skeleton, resulting in poikilocytes and fragmentation. Spectrin deficiency impairs the vertical interaction of the skeleton with the lipid bilayer and gives rise to microspherocytes as described for HS (Fig. 9-5).

Common Clinical Findings

The HE syndrome is heterogeneous in terms of clinical manifestations, ranging from an asymptomatic carrier state to homozygous HE and HPP with severe hemolysis presenting during infancy. Clinically, the most frequently occurring phenotype is mild HE with no or minimal hemolysis.¹¹ SAO heterozygotes are asymptomatic (Table 9-3).

Laboratory Testing and Results

Evidence of Hemolysis Typical HE patients present with a very mild, compensated hemolytic anemia in which the only features may be a slight reticulocytosis and decreased haptoglobin levels. Many patients show no biochemical evidence of a hemolytic process. In the more severe cases, such as HE with infantile poikilocytosis, homozygous HE, and HPP, the usual features of extravascular hemolysis outlined earlier are found.

Red Cell Indices In mild HE patients with compensated and uncompensated hemolysis, the MCV is usually normal or slightly elevated, the latter finding probably reflecting an associated reticulocytosis. MCH and MCHC are also usually within the normal range. In infants with HE and poikilocytosis, the MCV may be decreased and the MCHC is either normal or slightly elevated. In HPP, the MCV is always markedly decreased and the MCHC is usually elevated.

Morphology of Peripheral Smear The morphology of the peripheral smear varies with the clinical phenotypes of HE (Table 9-3). Asymptomatic carriers may have normal morphology. In mild HE with no hemolysis or a compensated hemolytic state, the RBC show prominent uniform elliptocytosis, the cells being elliptical rather than oval or egg-shaped (Fig. 9-8). Usually, more than 30% of the RBC are elliptocytic, but many patients have a higher proportion of up to 100% elliptocytes. Elongated or rod-shaped RBC are characteristic and often constitute more than 10% of the RBC. In patients with uncompensated hemolysis (mild HE with sporadic hemolysis), the RBC show more prominent poikilocytosis, and a small proportion of elliptocytes may have budlike projections. In mild HE with poikilocytosis of infancy there is initial prominent poikilocytosis, microspherocytosis, fragmentation, budding of red cells, and a variable degree of elliptocytosis (Fig. 9-11). By the time the infant reaches the age of 1 to 2 years, the morphology has changed to that characteristic of mild HE (Table 9-2). The rare patients with homozygous HE or HPP (Fig. 9-9) present with marked microspherocytosis, micropoikilocytosis, bizarre fragments, and very few, if any, elliptocytes. Patients with SAO have very distinctive red cell morphology. The elliptocytes are more rounded and oval, and

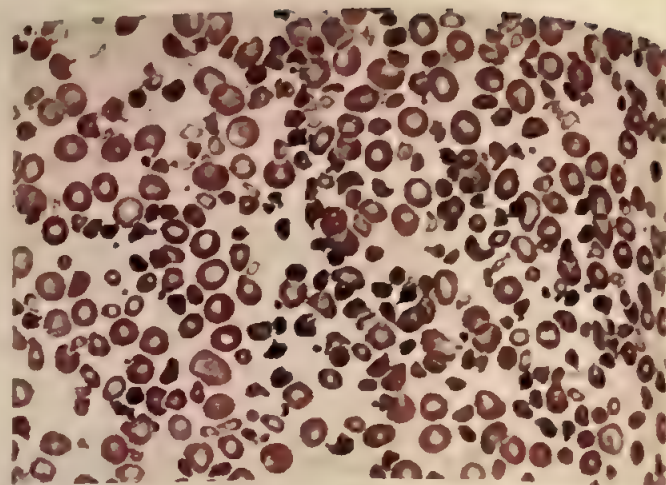


FIGURE 9-11 Photomicrograph of peripheral blood smear from a patient with mild hereditary elliptocytosis (HE) and poikilocytosis of infancy. Note the poikilocytosis and fragmentation.

are often quite large with a transverse bar that divides the central pale space (Fig. 9-10).

Special Laboratory Tests

Clinical laboratory results, family history, and the presence of elliptocytes or SAO ovalocytes are often sufficient to make a diagnosis, but specialized testing may be required in difficult cases.

RBC membrane protein deficiencies or size abnormalities may be detected by SDS-PAGE and quantitation, as described earlier for HS. Spectrin dimer self-association is analyzed by extracting spectrin from the membrane at 4°C followed by nondenaturing PAGE and quantitation of spectrin dimers and tetramers. Limited tryptic digestion of spectrin followed by SDS PAGE or two-dimensional gel electrophoresis will identify the defective domain. The EMA test shows a marked reduction in fluorescence in patients with HPP, and osmotic fragility is increased in severe HE and in HPP. The osmotic gradient ektacytometry profile is altered, particularly in HPP patients.

A suspected diagnosis of SAO is easily confirmed by detecting the presence of the characteristic heterozygous 27-base-pair deletion in the *SLC4A1* gene using the polymerase chain reaction (PCR). Research-oriented laboratories offer targeted NGS to identify the underlying gene mutations in HE and HPP and provide a molecular genetic diagnosis.

Treatment

Patients with compensated mild HE and no splenomegaly have a benign disorder and require no therapeutic intervention. Severe HE and HPP cases usually require blood transfusions and benefit from splenectomy. Patients with HE and infantile poikilocytosis should be recognized and treated symptomatically, because they will improve spontaneously with the development of a clinical picture indistinguishable from mild HE.

Disorders of Red Cell Hydration

Hereditary Hydrocytosis and Hereditary Xerocytosis

This is a heterogeneous group of rare disorders characterized by alterations in the permeability of the red cell membrane, which affects the level of hydration of the RBC. Two main clinical and morphological syndromes have been

described: hereditary hydrocytosis (stomatocytosis), in which the RBC are swollen (Fig. 9-12), and hereditary xerocytosis, in which the RBC are markedly dehydrated (Fig. 9-13). Several intermediate syndromes have been identified, but these are not considered here.

Inheritance and Epidemiology

Both disorders are inherited in an autosomal dominant fashion.

Molecular Defects and Pathophysiology

An important determinant of the water content of red cells is the total intracellular concentration of the monovalent cations sodium and potassium. To maintain osmotic equilibrium, water enters cells in which the total cation content is increased, leading to swelling and hydrocyte formation. In contrast, a net loss of cations results in a movement of water out of the cell with formation of dehydrated cells (xerocytes).

Overhydrated hereditary stomatocytosis, also known as hereditary hydrocytosis, is characterized by a marked passive sodium leak, which increases the sodium and water content of the cell and causes macrocytosis.^{11,31} In most patients there is either a lack or very low levels of stomatin, an integral RBC membrane protein. However, no gene mutations have been found, and stomatin knockout mice are normal. Causative heterozygous missense mutations have been documented in the transmembrane domain of Rhesus-associated glycoprotein (RhAG), which is a component of the AE1 macromolecular complex and functions as an ammonium and/or CO₂ transporter.

The hallmark of hereditary xerocytosis is a potassium leak from the red cells that is not accompanied by a proportional gain of sodium. Consequently, the net intracellular cation content and cell water content are decreased, and the RBC are dehydrated.^{11,31} It is a heterogeneous disorder typically caused by mutations in the *PIEZO1* gene or the *KCNN4* gene in a minority of patients. *PIEZO1* is a mechanosensitive cation

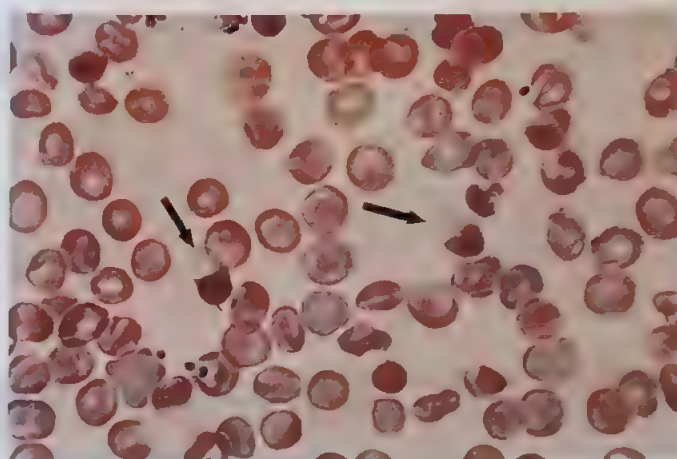


FIGURE 9-13 Photomicrograph of peripheral blood smear from a patient with hereditary xerocytosis. Note the characteristic target cells and cells with hemoglobin concentrated on one side of the cell, indicated by black arrows.

channel activated by mechanical stimuli encountered by RBC as they navigate narrow capillaries and splenic sinusoids. Most of the mutations are missense mutations that activate *PIEZO1* and increase cation efflux. *KCNN4* is a calcium-activated K⁺ channel, known as the Gardos channel, and mutations affect the channel kinetics.^{11,31}

Clinical Manifestations

Moderate to severe anemia is present in cases of hereditary hydrocytosis, and jaundice and splenomegaly are common features. Hereditary xerocytosis patients show significant clinical variation but typically present with mild to moderate compensated anemia, often with splenomegaly. In both disorders, there is a tendency toward iron overload regardless of transfusion history.

Laboratory Testing and Results

Red Cell Indices The MCHC is decreased in hereditary hydrocytosis, and the MCV is elevated up to 150 fL in severe cases, reflecting macrocytosis. In hereditary xerocytosis, the MCHC is increased, reflecting cellular dehydration, and the MCV is frequently mildly increased, possibly due to an elevated reticulocyte count.

Morphology of Peripheral Smear The characteristic morphological features of hereditary hydrocytosis are a tendency toward macrocytosis and the presence of stomatocytes and occasional spherocytes on the peripheral smear. Stomatocytes are RBC with a central slit or stoma and a bowl-like appearance (Fig. 9-12). In hereditary xerocytosis, target cells are present, reflecting the greater surface-to-volume ratio of these cells. Small spiculated echinocytes and cells with hemoglobin concentrated in one part of the cell (puddled) are also features of hereditary xerocytosis (Fig. 9-13).

Special Laboratory Tests

RBC sodium concentration is markedly elevated and potassium concentration is decreased in hereditary hydrocytosis. Total monovalent cation content is increased. Osmotic fragility is increased due to the decreased surface-to-volume ratio and many of the hydrated RBC approach their critical hemolytic volume and have limited deformability. The osmotic gradient

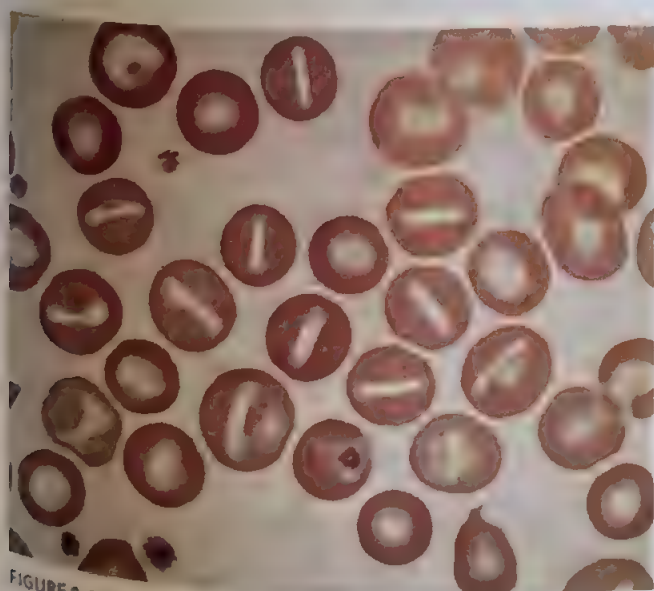


FIGURE 9-12 Photomicrograph of peripheral blood smear from a patient with hereditary hydrocytosis (stomatocytosis). Note the high percentage of red cells with a central slit of pallor. (From Bell, A. Hematology. In: Listen, Look and Learn. Bethesda, MD: Health and Education Resources, Inc. with permission.)

ektacytometer profile is shifted to the right, reflecting overhydration. In contrast, RBC in hereditary xerocytosis have a markedly decreased potassium concentration, whereas sodium concentration may be normal or increased, and total monovalent cation concentration is slightly reduced. Xerocytes are resistant to osmotic lysis, reflecting the increased surface-to-volume ratio. The osmotic gradient ektacytometry profile is shifted to the left indicative of cellular dehydration and reduced deformability.

Treatment

Hemolysis is reduced in hereditary hydrocytosis patients after splenectomy, but this procedure increases the risk of thromboembolic complications and pulmonary hypertension. Patients with hereditary xerocytosis do not benefit from splenectomy, presumably because of more generalized sequestration of these cells. There is a high risk of postsplenectomy thromboses and thus splenectomy is not recommended for this disorder.

SUMMARY CHART

- Hemolytic anemias are caused by intracorporeal or extracorporeal defects.
Hereditary abnormalities of the red cell membrane are intracorporeal defects.
- The most frequently used tests reflecting increased red cell destruction is the serum unconjugated (indirect) bilirubin and serum haptoglobin determinations.
- A reticulocyte production index (RPI) greater than 2.5 is indicative of hemolysis.

The RPI is calculated by the following equation:

$$\text{RPI} = \frac{\% \text{ Reticulocytes}}{\text{Reticulocyte maturation time}} \times \frac{\% \text{ Hematocrit}}{45\%}$$

- A systematic step-wise approach is optimal to diagnose the type of hemolytic anemia.
- The red cell membrane skeleton consists of structural proteins, α - and β -spectrin, which form heterotetramers that are linked together in horizontal interactions by actin and protein 4.1R.

The major integral red cell membrane protein is the anion exchanger 1 (AE1).

Ankyrin is the main linker protein connecting the skeleton to the phospholipid bilayer by vertical interactions with β -spectrin and AE1.

- The main hereditary hemolytic anemias due to red blood cell membrane protein defects are:
 1. Hereditary spherocytosis (HS)
 2. Hereditary elliptocytosis (HE) including hereditary pyropoikilocytosis (HPP) and Southeast Asian Ovalocytosis (SAO)
 3. Hereditary hydrocytosis and hereditary xerocytosis
- HS is caused by a deficiency of one of the membrane proteins involved in vertical interactions between the skeleton and the lipid bilayer.
- A combination of the EMA test and the acidified glycerol lysis test identifies the majority of HS patients.
- HE and HPP are mainly due to spectrin self-association defects that compromise horizontal interactions in the membrane.
SAO is caused by a 27 base pair in-frame deletion in the AE1 gene.
- Hereditary hydrocytosis and xerocytosis are disorders of membrane cation permeability which affect hydration of the red cell.
- Targeted next-generation sequencing (NGS) is useful to determine the gene mutations underlying the inherited red cell membrane disorders.

CASE STUDY 9-1

A 40-year-old woman presented to her physician with an attack of acute cholecystitis.

Physical examination revealed a palpable spleen in addition to the signs of acute cholecystitis. On investigation she was found to have numerous gallstones.

A routine blood count showed a mild, compensated hemolytic state: Hb, 13.8 g/dL; Hct, 38%; MCV, 80 fL; MCHC, 36.3 g/dL; reticulocyte count, 7%; polymorphs present; RPI, 3.9. The peripheral smear showed moderate numbers of spherocytes and a few microspherocytes. The Coombs' test was negative. Unconjugated bilirubin was 2.5 mg/dL, and the conjugated bilirubin was 0.5 mg/dL. Haptoglobin concentration was less than 10 mg/dL (normal range is 25 to 180 mg/dL).

Further investigation revealed a positive acidified glycerol lysis test and decreased fluorescence intensity after incubation of her RBC with eosin 5'-maleimide. After the acute episode had settled, an elective cholecystectomy was performed. A diagnosis of hereditary spherocytosis was made and confirmed in a subsequent study of the patient's family when two of her three children were found to have mild, compensated hemolytic states associated with spherocytosis. In view of the risk of recurrence of common bile duct calculi, an elective splenectomy was performed 6 months later, curing the hemolytic state.

CASE STUDY 9-1—cont'd**QUESTIONS**

1. Why was a Coombs' test performed on this patient?
2. Did the diagnosis of this patient follow the standard diagnostic protocol?
3. Which of the RBC indices is typically increased in hereditary spherocytosis (HS)?

ANSWERS:

1. A Coombs' test was performed on this patient to determine whether the hemolysis was due to an immunological process or a nonimmunologic process.
2. Yes, the diagnosis of this patient followed the standard diagnostic protocol. First, the CBC and bilirubin results confirmed that there was increased red blood cell destruction and production. The Coombs' test was used to determine whether hemolysis was due to an immunological process or not. The morphology confirmed presence of spherocytes. Confirmatory tests were used to definitively diagnose HS.
3. Mean corpuscular hemoglobin concentration (MCHC) is typically increased in hereditary spherocytosis (HS).

CASE STUDY 9-2

A 45-year-old woman presented to her physician complaining of malaise and tiredness on mild exertion. On physical examination, she was found to have slight scleral icterus and a two-finger splenomegaly. A blood count revealed the following: Hb, 11.0 g/dL; Hct, 32%; MCHC, 34.3 g/dL; MCV, 100 fL; reticulocyte count, 12.0%; shift cells on peripheral smear; and RPI, 5.7. The peripheral smear showed about 80% elliptocytes with some poikilocytosis consisting of a few fragmented cells and budding elliptocytes. Unconjugated bilirubin was 3.5 mg/dL, conjugated bilirubin, 0.6 mg/dL, and haptoglobin, 15 mg/dL (normal range is 25 to 180 mg/dL). EMA analysis was normal. Examination of RBC from the patient's family showed striking elliptocytes with normal hemoglobin and reticulocyte count in her father and in one of her three children. A diagnosis of mild HE with sporadic hemolysis was made, and a good response to splenectomy was obtained.

QUESTIONS

1. What parameter(s) is (are) suggestive of effective erythropoiesis?
2. Do the laboratory values presented here indicate hemolysis in this patient?
3. What is the significance of the RPI value?

ANSWERS

1. Reticulocyte count and RPI are suggestive of effective erythropoiesis.
2. Yes, the increased reticulocyte count and RPI result indicate increased RBC production. The increased bilirubin and decreased haptoglobin indicate increased RBC destruction. The presence of fragmented cells also indicates hemolysis.
3. The RPI is a more accurate assessment of the reticulocyte response to hemolytic anemia. It accounts for the presence of premature "shift" reticulocytes in the peripheral blood that have a longer maturation time.

CASE STUDY 9-3

A 6-year-old boy was noted by his mother to have slight scleral icterus and was referred for further investigation. He complained of some tiredness on exertion but was otherwise symptom-free. Physical examination showed only a one-finger splenomegaly. A blood count showed the following: Hb, 10.8 g/dL; Hct, 29%; MCHC, 37 g/dL; MCV, 100 fL; reticulocyte count, 10%. Numerous target cells, some spiculated cells, and a few cells showing eccentric concentration of hemoglobin at one pole of the red cell were seen on the peripheral smear. The unconjugated bilirubin level was mildly elevated, and serum haptoglobin levels were decreased. There was no hemoglobinemia or hemosiderinuria. The osmotic fragility curve was strikingly decreased. Determination of red cell cation concentrations revealed a markedly decreased red cell potassium level of 65 mEq/L of RBCs (normal is 90 to 104 mEq/L)

and a slightly elevated red cell sodium level of 15 mEq/L of RBCs (normal is 5 to 12 mEq/L). Similar findings were obtained in the child's father, who had previously been diagnosed at another center as having an "unusual" form of anemia. A diagnosis of hereditary xerocytosis was made. Splenectomy was not advised, and the child has maintained a hemoglobin level varying between 9.5 and 11.0 g/dL over the past 2 years.

QUESTIONS

1. How can this anemia be classified as indicated by the RBC indices?
2. What does the decreased osmotic fragility represent?
3. Why would a splenectomy not be beneficial in this case?
4. Why are these red cells (xerocytes) said to be dehydrated with regard to osmotic equilibrium?

Continued

CASE STUDY 9-3—cont'd**ANSWERS**

1. As indicated by the RBC indices, this anemia would be classified as normocytic, hypochromic anemia.
2. Osmotic fragility reflects the degree of RBC hemolysis that occurs when a sample of blood is subjected to osmotic stress, for example, a hypotonic saline solution. In this case, xerocytes are resistant to osmotic lysis, reflecting the increased surface-to-volume ratio.
3. Patients with hereditary xerocytosis do not benefit from splenectomy, presumably because of more generalized sequestration of these cells. There is a high risk of postsplenectomy thromboses and thus splenectomy is not recommended for this disorder.
4. RBC in hereditary xerocytosis have a markedly decreased potassium concentration, whereas sodium concentration may be normal or increased, and total monovalent cation concentration is slightly reduced.

REVIEW QUESTIONS

1. What happens when normal donor red cells are transfused into a patient with an intracorporeal red cell defect?
 - a. Donor cells are destroyed
 - b. Donor cells have normal survival
 - c. Depends on the severity of the defect
 - d. Depends on the severity of the anemia
2. Which of the following tests is not used to determine increased red cell destruction?
 - a. Unconjugated (indirect) bilirubin
 - b. Serum haptoglobin
 - c. EMA test
 - d. Lactate dehydrogenase (LDH)
3. An anemic patient investigated for a hemolytic state has the following laboratory findings: hemoglobin, 8 g/dL; hematocrit, 23%; reticulocyte count, 8%; polymorphs on peripheral smear. What is the RPI?
 - a. 8
 - b. 4
 - c. 2
 - d. 1
4. Which of the following tests are not used to assist in classifying the cause of RBC hemolysis?
 - a. Coombs' test
 - b. Morphology of peripheral smear
 - c. Osmotic gradient ektacytometry
 - d. Iron studies
5. Which red cell protein is not part of the membrane skeleton?
 - a. Protein 4.1R
 - b. Anion exchanger-1 (AE1)
 - c. Spectrin tetramers
 - d. Spectrin dimers
6. Which of the following RBC membrane protein deficiencies does not cause hereditary spherocytosis?
 - a. Ankyrin
 - b. Protein 4.1R
 - c. Protein 4.2
 - d. Anion exchanger 1(AE1)
7. Which of the following laboratory tests would not be typical of hereditary spherocytosis?
 - a. Increased osmotic fragility
 - b. Spherocytes on peripheral smear
 - c. Decreased MCHC
 - d. Increased RPI
8. Which is the most frequent functional abnormality affecting membrane skeleton proteins in hereditary elliptocytosis?
 - a. Defective binding of spectrin to ankyrin
 - b. Defective spectrin tetramer assembly
 - c. Defective binding of ankyrin to anion exchanger-1 (AE1)
 - d. Deficiency of protein 4.2
9. Which of the following abnormalities is thought to cause the severe fragmentation and microspherocytes characteristic of hereditary pyropoikilocytosis?
 - a. Susceptibility of spectrin to thermal denaturation
 - b. Membrane ankyrin deficiency
 - c. Unstable membrane lipids
 - d. Defective membrane spectrin tetramer assembly and spectrin deficiency
10. Which of the following findings are not typical of Southeast Asian Ovalocytosis?
 - a. A deficiency of anion exchanger-1 (AE1)
 - b. A deletion of nine amino acids in AE1
 - c. Resistance to infection by malaria parasite
 - d. Rigid spoon-shaped ovalocytes
11. Which disorder is classified as a disorder of membrane cation permeability?
 - a. Sideroblastic anemia
 - b. Hereditary hydrocytosis
 - c. Microangiopathic hemolytic anemia
 - d. Ehlers-Danlos syndrome

See answers at the back of this book.

Hemolytic Anemias

Intracorpuseular Defects: Hereditary Enzyme Deficiencies

Heather L. Phillips, PhD, MLS(ASCP)^{CM}, MLS(AMT)

CHAPTER OUTLINE

Enzyme Deficiencies: Hexose Monophosphate Pathway
Glucose-6-Phosphate Dehydrogenase Deficiency

Enzyme Deficiencies: Glycolytic Pathway
Pyruvate Kinase Deficiency

Other Enzyme Deficiencies of the Glycolytic Pathway

Enzyme Deficiencies: Methemoglobin Reductase Pathway
Methemoglobin Reductase Deficiency
Methemoglobinemia

Summary Chart
Case Study 10-1
Review Questions
References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 10-1** Name the most common glycolytic enzyme deficiency associated with the hexose monophosphate shunt or pentose phosphate pathway.
- 10-2** Name the most common glycolytic enzyme deficiency associated with the Embden–Meyerhof pathway.
- 10-3** Identify the red blood cell cytoplasmic inclusions associated with oxidative denaturation of hemoglobin.
- 10-4** List laboratory test results that would suggest a deficiency of glucose-6-phosphate dehydrogenase (G6PD).

- 10-5** Identify a laboratory test result that would indicate a pyruvate kinase (PK) deficiency.
- 10-6** Name the deficiency that causes hemoglobin to be oxidized from the ferrous to the ferric state.
- 10-7** Describe the clinical findings in G6PD deficiency.
- 10-8** Describe the clinical findings in PK deficiency.
- 10-9** Differentiate glycolytic enzyme deficiencies from RBC membrane defects and hemoglobinopathies.
- 10-10** Differentiate between acquired and inherited methemoglobinemia.

In 1926, 72 plantation workers in Panama suffered acute hemolysis after receiving the antimalarial drug 8-aminoquinoline. Subsequent reports from widely scattered geographic locations added credence to the relationship of hemolysis, cyanosis, and methemoglobinemia with the ingestion of certain antimalarial drugs. In 1953, Dacie and associates¹ evaluated apparently heterogeneous cases of congenital hemolytic anemias that had several common characteristics. There was no detectable abnormal hemoglobin, the antiglobulin test result was negative, the osmotic fragility test was normal, and there were no spherocytes seen on the peripheral smear. The term *hereditary nonspherocytic hemolytic anemia (HNSHA)* was used to describe the group, which was later found to be associated with red blood cell enzyme abnormalities. Biochemical and molecular studies rapidly advanced the further characterization of these anemias.

The most common anemia in this group is caused by the deficiency of **glucose-6-phosphate dehydrogenase (G6PD)**, an enzyme in the hexose monophosphate shunt, or pentose phosphate pathway. The second most frequent enzyme deficiency is that of **pyruvate kinase (PK)**, an essential enzyme in the Embden–Meyerhof glycolytic pathway. Many other enzyme deficiencies of these pathways have also been identified. They are variably associated with HNSHA. Traditionally, diagnosing the enzymopathies relied heavily on both clinical and laboratory findings. Today, molecular technologies are used as a first-line diagnostic tool to identify germ line mutations. Laboratory testing is directed toward identification of the specific enzyme deficiency. This chapter describes the key enzymes critically involved in well-known metabolic pathways such as the glycolytic and pentose phosphate pathway.

Enzyme Deficiencies: Hexose Monophosphate Pathway

Glucose-6-Phosphate Dehydrogenase Deficiency

Glucose-6-phosphate dehydrogenase deficiency is the most common red cell enzyme disorder worldwide. It is estimated more than 500 million people are affected worldwide by the G6PD mutation. It is widely known as the most common inherited enzyme defect. A pathological disorder linked to the ingestion of the Fava Bean (*Vicia faba*) has been identified for centuries. Pythagoras, a Greek philosopher and mathematician in 5th century BC, banned the ingestion of the fava bean by his followers due to identified pathological effects. In the early 1900s, several physicians located in southern Italy and Sardinia identified a series of pathological effects caused by eating fava beans and coined the term **favism** to describe its effects.² Carson and associates³ identified enzyme G6PD deficiency in 1956 in an individual who developed hemolytic anemia after the administration of the antimalarial drug primaquine (8-aminoquinoline). The enzyme was first purified from human red cells in 1966.² Further progress characterized the diverse variants of G6PD by sequencing of amino acids, cloning of cDNA, and sequencing of nucleotides.

More than 400 biochemical variants of G6PD enzyme have been described.⁷ Recent advances in molecular biology have enabled classification of the variants into more than 50 gene mutation groups.^{4,5} A deficiency can be caused when there is a reduction in the volume of enzyme molecules present or a structural change in the enzyme causes a qualitative change. The deficiency is usually caused by the instability of the G6PD enzyme caused by amino acid substitutions.⁵ G6PD is a vital component in all cells. In erythrocytes, the enzyme operates at 1-2% of its maximum potential even when put under oxidative stress. G6PD has a large volume of reductive potential that does not exist in individuals with a deficiency.⁶

The World Health Organization (WHO) organizes the varying degrees of G6PD deficiency into five classes.⁷ The class I variant will have less than 10% normal enzymatic activity with chronic hemolytic anemia. The class II variant will have severe enzyme deficiency with intermittent episodes of acute hemolysis. The class III variant will have 10% to 60% normal enzymatic activity with intermittent episodes of acute hemolysis.⁷ The class IV variant will feature no enzymatic deficiency or hemolysis. Lastly, the class V variant will display increased enzymatic activity.⁷

Mode of Inheritance

G6PD deficiency is transmitted by a mutant gene located on the X chromosome.⁶ This gene is located on the telomeric region of the X chromosome at the Xq28 locus.⁶ Hemizygous males carrying G6PD mutations on their only X chromosome and females homozygous with the mutant gene will exhibit full expression, inhibiting the source of NADPH able to catalyze the pentose phosphate pathway. Females heterozygous on the X chromosome with G6PD mutations can result in two red blood cell populations peripherally. One cell population will be G6PD deficient while the other cell population can be G6PD functional. This causes variation in enzymatic

activity and associated observed conditions. The expression of G6PD deficiency varies markedly among heterozygotes, which is explained in part by the X-inactivation hypothesis.⁸ In females, one of the two X chromosomes (maternally or paternally derived) becomes randomly inactivated in each cell of the early embryo. Thus, each somatic cell in a heterozygote expresses either one or the other G6PD allele (Gd). The ratio of the two cell types may vary widely, not only in different individuals but also among different tissues even within the same individual.⁸

The highest incidence of G6PD deficiency occurs in the darkly pigmented racial and ethnic groups, and the geographic distribution is primarily resigned to tropical, subtropical, and Mediterranean areas. However, G6PD variants can be found in the Middle East, North America, and northern European countries.⁵ The highest prevalence of G6PD deficiency is found in Africa and the Middle East with hot spots in the Mediterranean and Asia. While most locations exhibit a wide range of disease severity and varying distributions, the Kurdish Jews are the most affected population (60% to 70%), whereas the most severe variants are found in Mediterranean populations.⁵ People with African American and Mediterranean decent contain the highest prevalence of G6PD variants in the United States. In the African American population, 24% are carriers (heterogeneous) with normal levels of G6PD; subsequently, 10% to 14% of males express an abnormal variant.⁶ Of these African Americans, 1 out of 4 affected males have normal G6PD levels while 10% exhibit clinical abnormalities.^{5,7}

Normally active G6PD, type Gd B, is the most common form of the enzyme in all populations and exists in 99% of whites in the United States. Another variety of the G6PD enzyme, Gd A+, is commonly found in Africans, which has normal activity but differs from Gd B by a single amino acid substitution that alters its electrophoretic mobility.⁷ The Gd A+ variant is found in about 20% of African men.⁹ Among African Americans who possess the Gd A+ gene, there is a reduced activity variety, designated Gd A-, which can be demonstrated in 15% to 39% of the men.¹⁰ Gd A- is the prototype of the mild form of G6PD deficiency.⁷

Among whites, G6PD Mediterranean (G6PD Med) is the most common abnormal variant, although the overall prevalence is low. Among Kurdish Jews, however, the incidence of G6PD Med may be as high as 60% to 70%.⁷ G6PD Med (also known as G6PD B-) is the prototype of a more severe enzyme deficiency associated with acute hemolytic anemia, including favism.^{5,7} The variant Gd Canton is more commonly found in native people of Southeast Asia and China. There is a high frequency of G6PD deficiency in the Middle East.⁵ Approximately 16% of neonatal jaundice in Iraq is related to G6PD deficiency, whereas neonatal jaundice is rarely attributed to G6PD deficiency in the United States.¹¹ The type of G6PD variant found in selected populations is listed in Table 10-1.

The variants have been generally designated by geographic names. With the use of modern techniques of molecular biology, these variants have been reclassified in terms of the exact sites of nucleotide substitutions. Using this nomenclature, Gd A+ would be designated as G6PD A^{176G} to indicate the presence of guanine at nucleotide 376.¹²

TABLE 10-1 Distribution of Common G6PD Variants

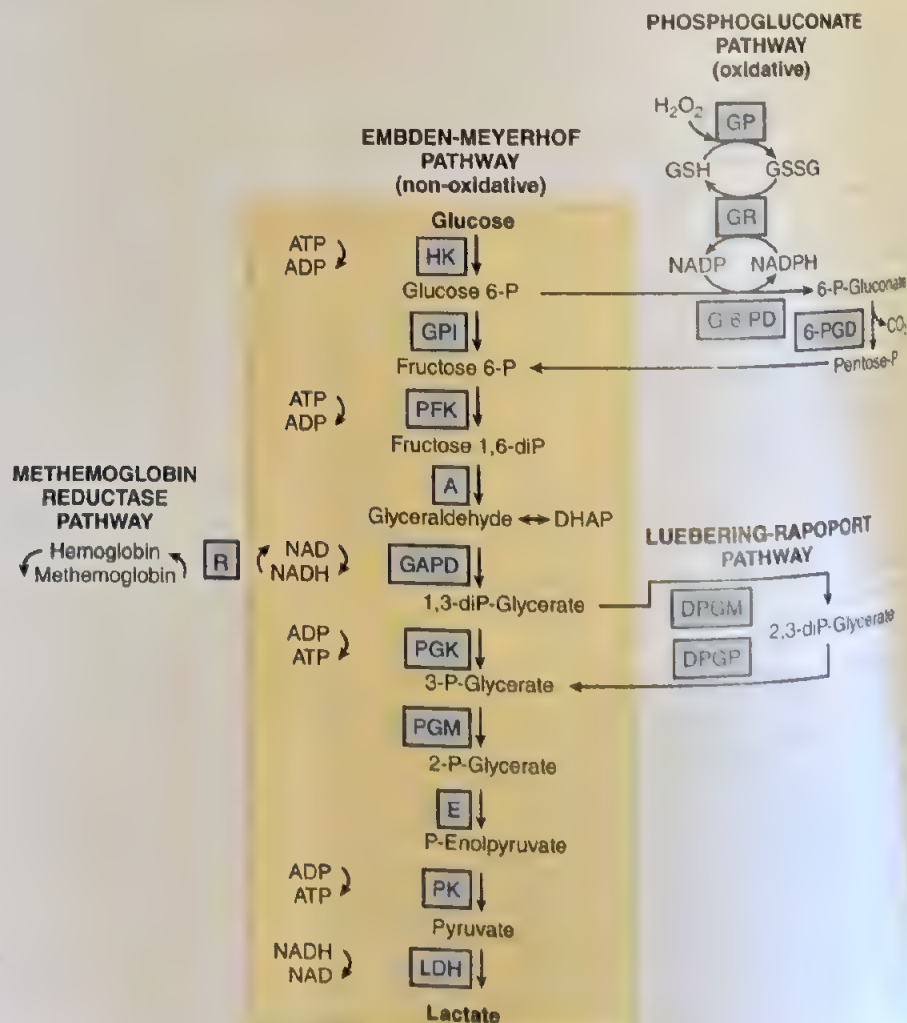
Enzyme Type	Population Usually Associated
Gd B (normal variant)	All
Gd Med (also known as Gd B-)	Whites (Mediterranean area)
Gd A+ (normal variant)	Blacks (~16% of African Americans)
Gd A-	Africans
Gd Canton	Asians

More than 310 variants have been identified involving the G6PD enzyme.¹² Most of these variants involve single base changes causing amino acid replacements. These single point mutations have been documented multiple times in varying geographic locations. This indicates the origin is unlikely to be from a common ancestor, and new mutations are independently emerging.¹² The most common G6PD mutations seen today are the Mediterranean variant 563C→T that exhibits a high frequency (97%) in the Middle East, Mediterranean, and Indian subcontinents.¹² African A- variant 202G→A is seen most often in the Arabian Mediterranean countries with 46% frequency seen in Algeria.¹²

Pathogenesis

G6PD is a cytoplasmic enzyme capable of catalyzing the first reaction of the **hexose monophosphate shunt** (or pentose phosphate) aerobic glycolytic pathway. This pathway has been called a shunt because it involves some reactions of the glycolytic pathway and therefore has been viewed as a shunt of glycolysis. Oxidative catabolism of glucose is accompanied by reduction of **nicotinamide adenine dinucleotide phosphate (NADP⁺)** to the extramitochondrial coenzyme **nicotinamide adenine dinucleotide phosphate (NADPH)** (Fig. 10-1). NADPH is required by erythrocytes, as it enables the counterbalance of oxidative stress triggered by several oxidative agents and protects the hemoglobin from oxidative denaturation. NADPH also allows for the preservation of the reduced form of **glutathione (GSH)**. GSH is essential to reduce hydrogen peroxide and oxygen radicals. Reduced glutathione is also used for hemoglobin maintenance and other proteins. Lacking mitochondria, the pentose phosphate pathway is the erythrocytes' only available source of NADPH. For the erythrocyte without G6PD, oxidative stress resulting in lysis is imminent. The sequence of biochemical reactions shown in Figure 10-2 occur within the normal RBC with adequate levels of appropriate enzymes and substrate to prevent the accumulation of intracellular oxidants.

FIGURE 10-1 Red cell metabolic pathways. The nucleated red cell depends almost exclusively on the breakdown of glucose for energy requirements. The Embden-Meyerhof (nonoxidative or anaerobic) pathway is responsible for most of the glucose utilization and generation of ATP. In addition, this pathway plays an essential role in maintaining pyridine nucleotides in a reduced state to support methemoglobin reduction (the methemoglobin reductase pathway) and 2,3-bisphosphoglycerate synthesis (the Luebering-Rapaport pathway). The phosphogluconate pathway couples oxidative metabolism with pyridine nucleotide and glutathione reduction. It serves to protect red cells from environmental oxidants.



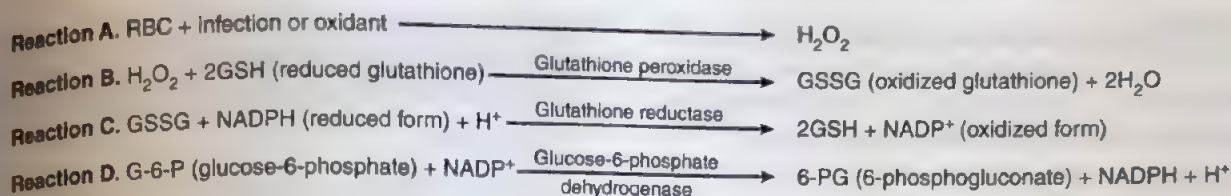


FIGURE 10-2 Reactions with erythrocytes to prevent accumulation of oxidants.

The activity of G6PD is highest in young erythrocytes and decreases with cell aging. Under normal conditions, the individual with G6PD deficiency compensates for the shortened life span of the erythrocytes by producing more early red cells (reticulocytosis). Oxidative stress, however, can lead to a mild to severe hemolytic episode. A deficiency of GSH results in oxidative destruction of certain erythrocyte components, including sulfhydryl groups of globin chains and the cell membrane.¹³ In addition, more than 50 chemical agents may induce hemolysis in G6PD-deficient erythrocytes. The drugs that have more commonly been reported to induce hemolysis in individuals with G6PD deficiency are listed in Box 10-1.

The hemolytic episode results when G6PD-deficient erythrocytes fail to maintain adequate levels of GSH.¹³ The resulting oxidation of hemoglobin leads to progressive precipitation of irreversibly denatured hemoglobin (Heinz bodies) (Fig. 10-3). The cells lack normal deformability when sulfhydryl groups are oxidized and consequently encounter difficulties passing through the microcirculation. Premature destruction of the cells results when they undergo intravascular lysis or are sequestered and destroyed in the liver and spleen. This early destruction may sometimes be detected in the peripheral blood smear with the formation of small, condensed, bite- or helmet-shaped red cells (Fig. 10-4).



FIGURE 10-3 Heinz bodies, using peripheral blood from a patient with G6PD deficiency, stained with the supravital stain, crystal violet.

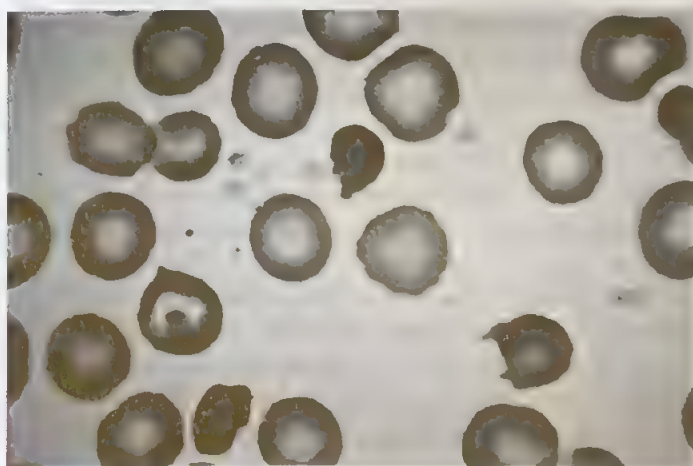


FIGURE 10-4 Peripheral blood smear from a patient with a G6PD deficiency. Note the small, condensed "bite" or "helmet" cells.

BOX 10-1 Drugs and Chemicals Associated with Hemolytic Anemia in G6PD Deficiency

- | | |
|--------------------------------|-------------------------|
| • Acetanilide | • Pamaquine |
| • Cotrimoxazole | • Pentaquine |
| • Dapsone | • Phenazopyridine |
| • Doxorubicin | • Phenylhydrazine |
| • Furazolidone | • Primaquine |
| • Methylene blue | • Rasburicase |
| • Moxifloxacin | • Sulfacetamide |
| • Nalidixic acid | • Sulfanilamide |
| • Naphthalene | • Sulfapyridine |
| • Niridazole (Ambilhar) | • Thiazolesulfone |
| • Nitrofurantoin (Furadantoin) | • Toluidine blue |
| • Norfloxacin | • Trinitrotoluene (TNT) |

Source: National Organization for Rare Disorders (NORD). Rare Disease Database: Glucose-6-Phosphate Dehydrogenase Deficiency. Available at <https://rarediseases.org/rare-diseases/glucose-6-phosphate-dehydrogenase-deficiency/>. Note. The exact degree of susceptibility to a drug varies from one person to another.

ADVANCED CONTENT

The fava bean contains up to 2% of beta-glucosides.¹⁴ Consumption of fava beans when the cells are G6PD deficient causes the pyrimidine glycosides, vicine and convicine, to undergo hydrolysis during digestion.¹⁴ This further causes the release of divicine and isouramil, responsible for increasing the activity of the hexose monophosphate shunt.¹⁴ This increased activity promotes the erythrocyte hemolysis seen in G6PD-deficient patients.

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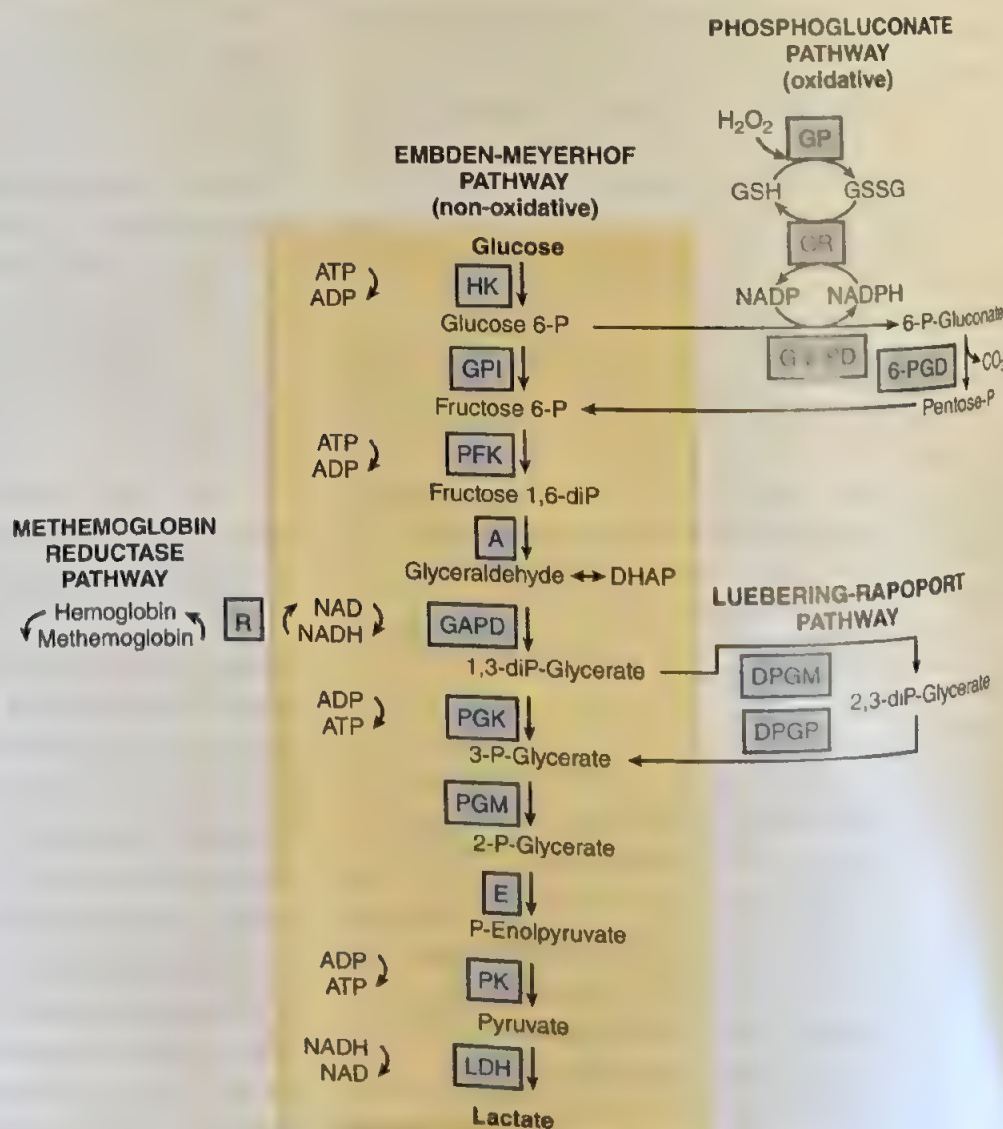
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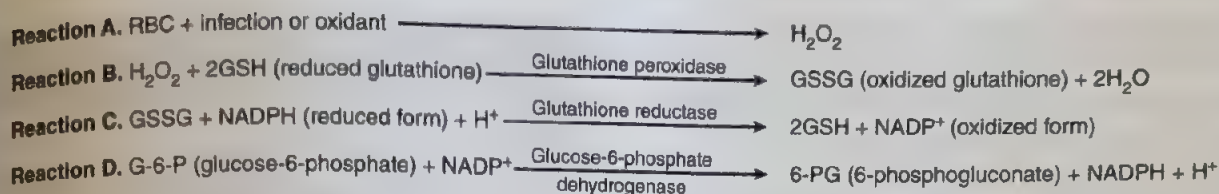


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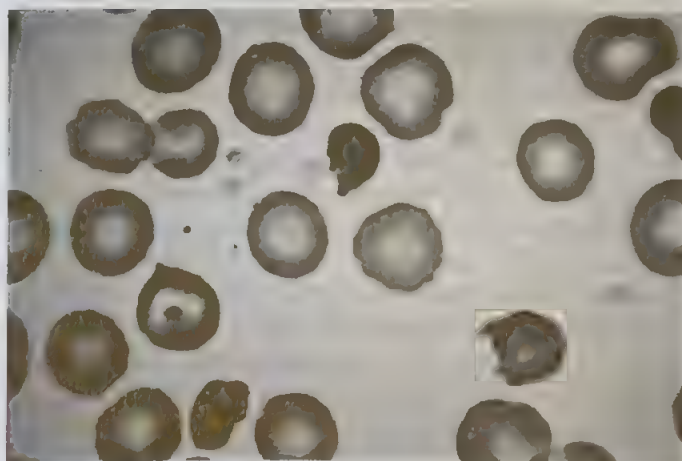


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| • Doxorubicin | • Phenylhydrazine |
| • Furazolidone | • Primaquine |
| • Methylene blue | • Rasburicase |
| • Moxifloxacin | • Sulfacetamide |
| • Nalidixic acid | • Sulfanilamide |
| • Naphthalene | • Sulfapyridine |
| • Nitrofurantoin (Ambilhar) | • Thiazolesulfone |
| • Nitrofurantoin (Furadantin) | • Toluidine blue |
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ADVANCED CONTENT

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Source: National Organization for Rare Disorders (NORD). Rare Disease Database: Glucose-6-Phosphate Dehydrogenase Deficiency. Available at <https://rarediseases.org/rare-diseases/glucose-6-phosphate-dehydrogenase-deficiency/>. Note: The exact degree of susceptibility to a drug varies from one person to another.

Hemolytic anemia that occurs in G6PD deficiency after the consumption of fava beans is referred to as favism (Fig. 10-5). Favism is found in some individuals with G6PD deficiency of the Mediterranean and Canton types.¹⁴

Clinical Findings

Most individuals with G6PD mutations are asymptomatic. If symptoms manifest from the mutation, it is likely in the form of hemolytic anemia brought on by oxidative stress.¹⁵ Under ordinary circumstances, it is possible for erythrocytes to survive and function normally with only 20% G6PD enzymatic activity.⁸ Newborn babies with severe neonatal jaundice and Mediterranean or African ancestry should be tested for G6PD deficiency. Neonate jaundice presents similar to physiological jaundice in 1 to 4 days.¹¹ This jaundice presentation is later than what is typically seen in blood group alloimmunization. The mechanism of action causing rapid jaundice in the neonate after delivery is not completely understood. Adults with chronic nonspherocytic hemolytic anemia should also be tested.

Presenting symptoms of the disorder are related to the severity of the acute or chronic hemolytic episode. After the administration of the offending drug, the erythrocyte count will decrease 2 to 3 days later due to lysis. This ultimately causes a rapid decrease in hemoglobin and hematocrit values. The anemia appears as normochromic and normocytic with an increase in the reticulocyte count. The patient may experience back pain. Hemoglobinuria and jaundice may also be evidence of the hemolytic process. The clinical features of the two most common variants are compared in Table 10-2.

Acute hemolysis is clinically indicated by anemia, jaundice, fatigue, backpain, increased unconjugated bilirubin, increased lactate dehydrogenase, splenomegaly, and an increase in reticulocytes in G6PD deficiency. A peripheral blood smear stained with a Romanowsky stain will indicate the presence of Heinz bodies and bite cells. The patient's urine may be dark, indicating hemoglobinuria. Upon microscopic observation, hemoglobin casts may be seen if acute tubular necrosis occurs.

The hemolytic episode in Gd A- is usually self-limiting.¹³ Young cells that are produced in response to the anemia have levels of G6PD that are nearly normal and have better survival characteristics. Hemolysis associated with G6PD Med is more easily induced, usually more severe, and has been reported to

TABLE 10-2 Comparison of Clinical Features of Gd A- and Gd Med (Gd B-)

Clinical Feature	Gd A-	Gd Med
Cells affected by defect	Aging erythrocytes	All erythrocytes
Hemolysis with drugs	Unusual	Common
Hemolysis with infection	Common	Common
Favism	No	Occasionally
Degree of hemolysis	Moderate	Severe
Transfusions required	No	Occasionally
Chronic hemolysis	No	No
Hemolytic disease of newborn	Rare	Occasionally

result in death on occasion.⁶ Red blood cell transfusions may be indicated for hemolytic episodes in patients with G6PD Med.⁴

Hemolysis of peripheral erythrocytes can be triggered by exogenous agents such as infections, consumption of *Vicia faba* (fava beans), drugs causing high-oxidative reduction potential (such as the antimalarial agent, primaquine), or any metabolic disturbances. Other clinical manifestations can perpetuate hemolysis, such as myocardial infarction and diabetes. Strenuous exercise has also been documented to cause increase hemolysis in patients with G6PD deficiency. The precise mechanism by which an increase in sensitivity among other clinical manifestations and the amount of oxidative stress that leads to hemolysis is unknown.¹⁵ Chronic hemolysis in G6PD deficiency can be exasperated by coinherited erythrocyte alterations, such as pyruvate kinase deficiency, thalassemia, and other inherited anemias.^{16,17}

CRITICAL THINKING QUESTION

10-1 G6PD deficiency can cause which type of hemolysis in affected individuals and what peripheral finding indicates this?

See answers to all Critical Thinking Questions at the back of this book.

Treatment for hemolytic anemia often involves erythrocyte transfusion when the hemoglobin and hematocrit fall to dangerous levels. Identifying disease-causing mutations can prove challenging in patients who have undergone extensive transfusions because of the presence of two populations of RBCs.

Laboratory Testing and Results

Testing has shifted since the discovery of hereditary deficiency anemia. Initially, clinical manifestations were coupled with laboratory results to obtain the most accurate diagnosis available for the time. Laboratory diagnosis eventually shifted to the use of biochemical markers as a means to properly diagnose patients. Today, gene sequencing and other molecular techniques are used to obtain diagnostic results to effectively treat symptomatic patients.



FIGURE 10-5 Fava beans.

G6PD deficiency should be suspected after a clinical episode of acute hemolysis after administration of chemical or therapeutic agents known to cause the reaction in patients with the disorder. Diagnosis of G6PD deficiency hinges on the total estimated enzyme activity. Quantitative spectrophotometric analysis can be used to determine the concentration of G6PD by measuring the rate of production of NADPH from NADP.

Laboratory changes of the nonspecific type include a fall in hemoglobin (and hematocrit), hemoglobinuria (urine can turn brown to almost black secondary to the presence of hemoglobin), Heinz bodies in the erythrocytes, evidence of hemolysis in the serum, elevated serum bilirubin levels, and markedly decreased or absent haptoglobin levels. Generally, there are no significant alterations in leukocyte or platelet counts or function.

Laboratory investigation of hemolytic anemia when there is evidence (family history, drug sensitivity, or both) of G6PD deficiency may include several screening procedures. The oxidative denaturation of hemoglobin results in the formation of Heinz bodies. In individuals with G6PD deficiency, the bonds between the heme and globin are oxidized. The heme is recycled, and the globulin chains are denatured forming a ball that sticks to the outer membrane of the erythrocyte. These small particles of precipitated denatured globulin chains can be visualized by supravital staining using certain basic dyes such as crystal violet (see Fig. 10-3). Heinz bodies will appear as small (1- to 4- μ m) purple inclusions, usually seen on the cell periphery. They are not seen with Romanowsky stains such as Wright's stain, preventing them from being seen on a peripheral thin prep slide. Although Heinz bodies may be seen in some other enzyme deficiencies, some unstable anemias are capable of forming Heinz bodies after the incubation of erythrocytes at 37°C for 48 hours.

Other test procedures that may be used to screen for G6PD deficiency include the methemoglobin reduction test and the ascorbate-cyanide test.¹³ In the methemoglobin reduction test, which is a simple and sensitive screening procedure, G6PD-deficient erythrocytes fail to reduce methemoglobin in the presence of methylene blue. The ascorbate-cyanide test, which measures peroxidative denaturation of hemoglobin, is not specific for G6PD deficiency, because it will yield moderately positive results if the patient has PK deficiency or certain unstable anemias. In addition, the fluorescent spot test and the specific G6PD assay are positive only with G6PD deficiency.¹¹ When a mixture of glucose-6-phosphate, NADP, saponin, and buffer is mixed with blood and placed on filter paper, G6PD converts the NADP to its reduced form, NADPH. When the filter paper is observed under fluorescent light, those erythrocytes that fail to convert NADP to NADPH (i.e., are deficient in G6PD) will lack fluorescence. Flow cytometry based on classic cytochemical staining of reduced tetrazolium salts has been successful in differentiating G6PD from other disorders. Unfortunately, it is both labor intensive and time consuming. The quantitative assay of G6PD is based on the measurement of the rate of reduction of NADP to NADPH measured at 340 nm and is the gold standard for G6PD diagnosis.¹⁸ G6PD variants can also be identified via electrophoretic methods.

Heterozygous females contain two cell populations peripherally, causing varying degrees of functional G6PD enzymatic activity. A single blood sample should contain both cell populations. Testing a heterogenous sample will provide an average of both deficient and sufficient red cell populations. This can provide falsely normal readings when biochemical assays are used. As many as 50% of heterozygous females are misclassified as "normal" when biochemical testing is performed.^{18,19} Currently, there is no reliable biochemical assay to detect G6PD heterozygotes. When testing at-risk heterozygous populations, a DNA analysis should be performed to correctly identify a G6PD deficiency.

False results may also occur when attempting to determine the concentration of G6PD enzymatic activity during an acute hemolysis episode. Elevations in reticulocytes or testing of neonates may also produce false results, as immature erythrocytes produce greater amounts of G6PD than mature cells.²⁰

Common mutations can be identified using restriction enzyme analysis after PCR (polymerase chain reaction) amplification.

Enzyme Deficiencies: Glycolytic Pathway

Several hereditary enzyme deficiencies have been described in the RBC glycolytic pathway (Embden-Meyerhof glycolytic pathway). Most of these glycolytic enzyme deficiencies are inherited autosomal recessive and do *not* demonstrate any abnormal RBC morphology. This differentiates them from RBC membrane defects and hemoglobinopathies, which do have specific abnormal RBC morphology associated with the specific disease (see Chapters 9 and 11). These inherited glycolytic enzyme deficiencies generally cause **chronic nonspherocytic hemolytic anemia (CNSHA)**, which vary in the degree of severity. The ability to compensate for anemia caused by the hemolysis is reflected in the increased production of RBCs in the bone marrow and reticulocytosis. Reticulocytes still have cytoplasmic organelles and are capable of protein synthesis and oxidative phosphorylation leading to ATP production. Several enzymes including pyruvate kinase (PK), hexokinase (HK), and aldolase have a much higher activity in reticulocytes and are often termed the "age-related" enzymes.²¹

Pyruvate Kinase Deficiency (PKD)

Pyruvate kinase deficiency is a rare congenital hemolytic anemia with a diverse phenotype and wide spectrum of severity. In 1954, Selwyn and Dacie observed a connection between defective glycolysis and hemolytic anemia. They identified a relationship between patients with congenital nonspherocytic hemolytic anemia (CNHSA) and an increased rate of *in vitro* hemolysis when defibrinated blood was incubated in the presence of glucose. Varying degrees of hemolysis patterns were reported. When tested in the lab, some samples demonstrated a reduction in the rate of autohemolysis when ATP was added to samples from patients with decreased erythrocyte ATP and increased 2,3 bisphosphoglycerate (2,3-BPG).²¹

In 1960 DeGruchy and associates²² reported that some patients with hereditary nonspherocytic hemolytic anemia (HNSHA) had elevated RBC concentrations of 2,3-BPG. This elevation suggested a block in anaerobic glycolysis further down the pathway (see Fig. 10-1). Multiple families exhibiting

CNSHA were assayed for the erythrocytic activity of glycolytic enzymes.²³ The enzyme was identified in 1961 when a severe deficiency of red blood cell pyruvate kinase (PK) was found in three patients with HNSHA.²³ PK catalyzes one of the reaction steps in the Embden–Meyerhof pathway of anaerobic glycolysis. Pyruvate kinase is a key regulator in glycolysis through the conversion of phosphoenolpyruvate (PEP) into pyruvate.

Because mature red blood cells lack mitochondria, they are dependent on anaerobic glycolysis for the generation of adenosine 5'-triphosphate (ATP). The diminished capacity to generate ATP in PK-deficient red blood cells results in cell membrane fragility and a hemolytic anemia.²⁴

Since its discovery, more than 300 pyruvate kinase gene (*PKLR*) mutations have been attributed to PKD, and many of these were cases of variant enzymes with different biochemical characteristics.²⁵ The nucleotide sequence of cDNA for the human PK gene and sequence of several of the mutations that cause HNSHA have been described.²⁵ PK deficiency is the most common enzymatic disease involving the anaerobic glycolytic pathway of the red blood cell. Together, G6PD deficiency and PK deficiency constitute the majority of HNSHA cases arising from erythrocyte enzyme deficiencies.

ADVANCED CONTENT

Pyruvate kinase deficiency has proven difficult to diagnose.^{26,27} Autosomal recessive erythrocyte disorders are likely not indicated by family history. Hemolytic disorders often require transfusions for supportive therapy. Mixed cell populations, PK deficient patient cells with healthy donor cells, cause an incorrect interpretation of biochemical tests. The Human Genome Mutation Database (HGMD) and a recent study of over 250 PKD patients from around the world in the "Pyruvate Kinase Deficiency Natural History Study (PKD NHS)" continue to correlate and add to the list of confirmed mutations of the *PKLR* gene.²⁵

Mode of Inheritance

Pyruvate kinase deficiency (PKD) demonstrates an autosomal recessive inheritance pattern encoding erythrocyte PK on the 1q21 chromosome.²⁸ True homozygotes are rare and restricted to children of consanguineous mating. The most common mode of inheritance is that of double heterozygosity. This is when two mutant variants of the PK enzyme are simultaneously inherited from each parent, making genotype-phenotype correlations challenging.^{27,28,29} There are now 371 *PKLR* gene variants associated with PKD.³⁰ The clinical symptoms of PKD are observed both in true homozygotes and in double heterozygotes for the PK gene.^{29,30}

ADVANCED CONTENT

Of the known mutations, 70% to 80% are missense substitutions. A missense mutation is when the change of a single base pair causes the substitution of a different amino

acid in the resulting protein. A nonmissense mutation is the substitution of a single base pair that leads to the appearance of a stop codon where previously there was a codon specifying an amino acid. The presence of this premature stop codon results in the production of a shortened, and likely nonfunctional, protein. Homozygotes express <25% of pyruvate kinase, whereas heterozygotes express 40% to 60% of pyruvate kinase.^{29,30} Some variants can retain normal or near-normal pyruvate kinase activity.³⁰ Heterozygote frequencies exist in 0.15% to 6% of varying populations.³⁰ Both sexes appear to be affected equally.

The PKD mutation exhibits a wide geographic distribution, with most of the cases reported to date in northern Europe, the United States, and Japan.³⁰ Other cases have been reported in Australia, Canada, China, Costa Rica, Hong Kong, Italy, Mexico, the Near East, New Zealand, the Philippines, Saudi Arabia, Spain, and Venezuela.^{29,30}

ADVANCED CONTENT

Inherited metabolic deficiencies have a wide geographic distribution, having the highest concentration in areas overlapping with geographic regions endemic with malaria. Research suggests evolutionary benefits to those individuals whose genes contain mutations for metabolic deficiencies that affect the erythrocyte.³⁰ Current research reports enhanced phagocytosis of the parasitized erythrocyte in individuals who have heterozygous or homozygous mutations for pyruvate kinase deficiency.³¹ The malarial parasite is unable to replicate in erythrocytes with a pyruvate kinase deficiency in mice.³² Inherited metabolic mutations may be evolving under the selective pressure of malaria.

PKD has a worldwide geographical distribution with an estimated prevalence ranging from 1 to 5 per 100,000 in the Caucasian population,³⁰ but this does not include the undiagnosed PKD. The Pennsylvania Amish have a higher frequency of PK deficiency, which has been traced back to a single immigrant couple.³⁰ In affected families, consanguinity is common. Thus, the PK deficiency in the Amish population is the result of a true homozygote condition.³⁰

Pathogenesis

The erythrocyte relies on glycolysis to generate adenosine triphosphate (ATP) for both its structural and functional integrity. In the erythrocyte, glucose catabolism occurs through the anaerobic Embden–Meyerhof pathway 90% of the time. Utilization of this pathway provides a source of ATP. 2,3-bisphosphoglycerate (2,3-BPG) and nicotinamide adenine dinucleotide (NADH).²¹ This organic phosphate, 2,3-BPG, is used for the modulation of hemoglobin oxygen affinity. NADH is used by the erythrocyte for enzymatic reduction of methemoglobin, necessary for cellular function.

Pyruvate kinase catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate. This step is vital for the erythrocyte as this generates the ATP required for cellular function. PK deficiency results in a decreased capacity to generate necessary ATP (see Fig. 10-1 and Chapter 2). The ATP-requiring membrane pumps that maintain the proper electrochemical gradients begin to fail with decreasing concentrations of ATP. This results in cell water loss with cell shrinkage, distortion of cell shape, and increased membrane rigidity.²¹ These membrane abnormalities lead to premature destruction of the red blood cells in the spleen and liver with consequent anemia. It has been shown that PK-deficient reticulocytes consume six to seven times more oxygen than normal reticulocytes.²⁹ In most cells, the drop in ATP regeneration because of a block in the glycolytic pathway would be compensated for by oxidative phosphorylation, but that capacity is lost in red blood cells as they mature and they lose mitochondria. Pyruvate kinase deficiency leads to ATP depletion, which affects the viability of the erythrocyte.

Inability to fully utilize glycolytic pathway intermediates required for successful completion of the glycolytic pathway leads to an accumulation of components such as 2-phosphoglycerate, 3-phosphoglycerate, 2,3-BPG and PEP.^{29,30} Accumulation of products such as 2,3-BPG further contributes to the impairment of the glycolytic pathway as it inhibits hexokinase. Increased 2,3-BPG causes a rightward shift in the oxygen association curve of hemoglobin in erythrocytes, allowing anemic states to be tolerated in those with mild pyruvate kinase deficiency.

Clinical Findings

The severity of symptoms of PKD is highly variable, ranging from mild anemia or fully compensated hemolysis to life-threatening anemia necessitating neonatal exchange transfusions. Individuals with PKD can be affected as early as during in utero development. In utero, infants with PKD are often affected by complications such as intrauterine growth restriction, prematurity, and hydrops fetalis.^{27,29} In the PKD Natural History Study, there was no association identified between the PK genotype and the frequency of complications in utero or in the newborn.²⁶ This suggests that the clinical presentation at birth is not predictable. However, all women with two nonmissense *PKLR* variants required transfusion support during pregnancy, whereas only 43% of women with two missense mutations required transfusions.³⁰ After delivery, 59% to 60% of neonates experience jaundice with severe indirect hyperbilirubinemia caused by significant hemolysis requiring transfusion.^{24,27}

Homozygous PKD patients seen in the Amish population presented with symptoms soon after birth leading to a rapid diagnosis in early infancy.³⁰ These patients had the highest rate of splenectomy (93%) and the highest rate of certain complications, including extramedullary hematopoiesis.³⁰

Children and adults with known pyruvate kinase deficiency also experience a wide range of symptoms and clinical manifestations, including chronic hemolytic anemia, jaundice, bilirubin gallstones, splenomegaly, and iron overload. Children who experience significant anemia may require regular transfusion support.³⁰

Symptoms experienced by those with pyruvate kinase deficiency are often exacerbated by acute infections, pregnancy, or stress.²⁴ Supplemental treatment for PKD often involves the removal of the spleen. Hemoglobin results before splenectomy often range from 6.5 to 11.0 g/dL. Postsplenectomy, hemoglobin often increased by 15 g/L.²⁴ In severe PKD, transfusions are common. Eighty-four percent of patients with PKD will have been transfused at least once in their lifetime.²⁴ Patients do experience iron overload regularly. Supportive therapy involves chelation of the iron from the body. As is true with all chronic hemolytic disorders, there is an increase incidence of pigmented gallstone formation in patients with PKD.^{27,29}

With an improved understanding of the relationship between genotype and clinical features, genetic testing has been recommended to assist in determination of expected prognosis and development of an individualized monitoring plan. Consensus guidelines on the diagnosis of PK deficiency recommend diagnostic confirmation with genetic testing.³⁰

Laboratory Testing and Results

The peripheral blood smears of patients with PK deficiency typically show a normochromic, normocytic anemia with varying degrees of reticulocytosis, ranging anywhere from 4% to 11%.²⁷ Spleens are often removed once a diagnosed child has reached the age of five years old. Postsplenectomy, reticulocytes increase anywhere from 20% to 70%.²⁷ Rapid increases in reticulocytes are due to longer peripheral circulation due to the removal of the spleen.

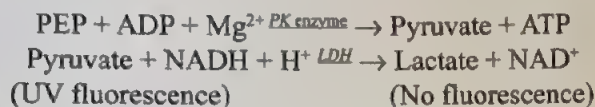
Ordinary erythrocyte morphology is usually demonstrated, but as many as 5% to 20% of echinocytes can be observed.²⁶ Erythroblasts may be observed on a peripheral blood smear in severe cases of PKD. Accelerated erythropoiesis may result in polychromasia, poikilocytosis, anisocytosis, and nucleated red blood cells. Both the hemoglobin and the hematocrit levels are below normal. The serum usually has a moderate increase in unconjugated bilirubin, and the haptoglobin level is decreased or absent.²⁶ Other results such as indirect hyperbilirubinemia will demonstrate results such as 60 $\mu\text{mol/L}$ and ferritin $>1,000 \mu\text{g/L}$ unless on a chelation protocol.^{26,27} PKD patients will likely exhibit normal lactate dehydrogenase, even though this is a marker often used for intravascular hemolysis.

Several screening tests may be used to distinguish the nonspherocytic anemia of PK deficiency from the anemias of hereditary spherocytosis and the unstable hemoglobinopathies.³³ These tests are nonspecific and serve only as a mechanism for classifying the type of anemia.

Screening tests may include the osmotic fragility test and the autohemolysis test (see Chapter 9 & 31), as well as the antiglobulin test and red blood cell survival tests. Erythrocytes that are PK-deficient have osmotic fragility, which is nearly normal when the test is performed on freshly drawn blood. If the blood is incubated for a few hours, some patients exhibit an increase in osmotic fragility.²¹ Sterile defibrinated blood is used to perform the test for autohemolysis. When normal erythrocytes are incubated in their own serum at 37°C, they gradually lyse, showing up to 3.5% lysis after 48 hours.²¹

Erythrocytes from patients with nonspherocytic anemias, as well as those with hereditary spherocytosis, demonstrate an increased amount of autohemolysis.³³ When glucose is added before incubation, erythrocytes from the patient with hereditary spherocytosis demonstrate a decreased amount of autohemolysis.²¹ The addition of glucose does not correct the increased autohemolysis of PK-deficient erythrocytes. The antiglobulin test in PK deficiency is negative, and the red blood cell survival is decreased.²¹

A fluorescence screening test can be used for the presumptive diagnosis of PK deficiency.³⁴ The test is based on the following coupled enzyme assay:



This assay takes advantage of the fact that NADH fluoresces when it is illuminated with long-wave ultraviolet (UV) light, whereas NAD does not fluoresce. Phosphoenolpyruvate (PEP), NADH, adenosine diphosphate (ADP), Mg^{2+} , and lactate dehydrogenase (LDH) are added to a patient sample of blood, which is spotted on filter paper and examined with a UV light. If the blood lacks PK enzyme, NADH will not be oxidized, and the fluorescence will persist for 45 minutes to an hour. If the blood is normal and has the PK enzyme, the fluorescence will disappear in 15 minutes, because NAD^+ does not fluoresce.³⁴ It should be noted that leukocytes contain a PK isoenzyme that will also catalyze the same reaction. Therefore, blood must be centrifuged and plasma and buffy coat removed before testing the erythrocytes.²¹ In addition, patients who have recently been transfused may have enough donor cells remaining in circulation to give erroneous test results.

Any abnormal fluorescence spot test should be followed with a confirmatory quantitative PK enzyme assay. This involves the same coupled reaction mechanisms as previously described, but the conversion of NADH to NAD^+ is measured spectrophotometrically at 340 nm under standard conditions. Most PK-deficient individuals have 5% to 25% of normal activity.^{34,35}

Spectrophotometric assays are used to determine the pyruvate kinase activity found in PKD patient erythrocytes. Pyruvate kinase enzyme testing, also known as biochemical testing, detects the quantifiable presence of pyruvate kinase activity in the erythrocyte. Interferences may occur causing false negative results. Recent transfusions or increased reticulocytes can cause false normal results. Patients should not be tested for pyruvate kinase activity until 40 days following a transfusion.^{21,34} Increased reticulocytes can also cause decreased activity to appear as a normal result.²¹ The younger the erythrocyte's age, the higher the pyruvate kinase activity.

CRITICAL THINKING QUESTION

10-2 What type of hemolysis is present in patients with PK deficiency, and how is this reflected in the peripheral blood smear?

ADVANCED CONTENT

Other factors may also contribute to the inaccuracy of results. Pyruvate kinase exists in various isoforms that can exhibit diverse biological functions and outcomes. The pyruvate kinase isoenzyme type M2 (PKM2) controls cell progression and survival through the regulation of key signaling pathways. Some erythrocytes may exhibit a compensatory expression of the M2 isoenzyme.³⁶

False-positive results may occur due to secondary pathologies, such as mutations of the Krüppel-like factor 1 (KLF1) gene. KLF1 is a transcription factor that globally activates genes involved in erythroid cell development. Various mutations in the human KLF1 gene have been identified in acute myeloid leukemias and myelodysplastic syndromes.³⁷

Mutant PK displaying kinetic abnormalities can exhibit a falsely normal or higher pyruvate kinase activity when tested in the laboratory. In vivo, these mutant PK do not exhibit such conditions. In such cases, thermal stability testing should be performed. Samples are incubated for 1 hour at 53°C to determine whether the pyruvate kinase is a dysfunctional thermolabile enzyme variant.

Pyruvate kinase activity results should be reported with units IU/gHb.^{34,37} No general reference exists for testing. This is largely due to the temperature dependency of testing. While it is recommended to test at 37°C, laboratories perform testing at various temperatures. When decreased pyruvate kinase activity is detected, genotyping of the erythrocyte pyruvate kinase gene (*PKLR*) should be performed to indicate the presence of a mutation and confirm PKD diagnosis. Today, diagnosis is made on the basis of biochemical or molecular genomic testing for the PK enzyme.

Molecular testing, although highly specific, is often ineffective when utilizing a targeted approach. Limitations of genetic analysis arise when mutations occur outside of the targeted area or when abnormal genes that interact with the *PKLR* gene are abnormal.³⁴

Other Enzyme Deficiencies of the Glycolytic Pathway
Although there have been reports of other enzyme deficiencies in the glycolytic pathway, not all such deficiencies have been associated with hemolytic anemia. Other reported glycolytic enzyme deficiencies are summarized in Table 10-3, and the prevalence and characteristics are listed.⁴³

Hexokinase Deficiency

Hexokinase (HK) is an important rate-limiting enzyme for the Embden-Meyerhof pathway, where the enzyme is responsible for catalyzing the phosphorylation of glucose to the high-energy glucose 6-phosphate by MgATP. Hexokinase isoenzymes HK-1 and HK-R are encoded by the same gene located on chromosome 10.^{40,41} HK-1 is expressed in platelets, lymphocytes, and fibroblasts.⁴⁰ HK-R is exclusively expressed in erythrocytes. Valentine et al. described the first case of hexokinase deficiency and its association with hemolytic anemia.

TABLE 10-3 Other Glycolytic Enzyme Deficiencies

Enzyme	Prevalence	Characteristics
Hexokinase deficiency	20 families described	Wide range of severity described from a compensated chronic hemolytic anemia to severe neonatal hemolysis and death. Splenectomy is generally beneficial to the patient.
Glucose-6-phosphate isomerase deficiency (GPI)	60 families described Second in frequency to PK deficiency	Chronic hemolytic anemia of variable severity. Viral or bacterial infections can precipitate hemolytic crises. Hydrops fetalis is more common in GPI deficiency than in any other enzyme deficiency.
Phosphofructokinase deficiency	100 patients described worldwide. Approximately one third of the patients are of Jewish descent.	Patients may exhibit a mild hemolytic anemia with or without myopathy depending on the RBC PFK isoenzyme deficiency.
Aldolase deficiency	Very rare, 6 deficiencies in 5 families described.	Moderate chronic hemolytic anemia, mental retardation, and myopathy.
Triosephosphate isomerase deficiency (TPI)	40 cases reported	Neonatal hemolytic anemia with hyperbilirubinemia (usually requiring an exchange transfusion), progressive neurological dysfunction, cardiomyopathy, and infection. Most individuals die in childhood before 6 years of age.
Phosphoglycerate kinase deficiency	33 families reported	Chronic hemolytic anemia of varying severity, myopathy, and CNS dysfunction

1967.³⁹ HK deficiency is a rare autosomal recessive disorder. Currently, only 24 cases of hexokinase deficiency have been documented with six known mutations.⁴² Due to the enzyme's crucial role in glycolysis, a complete loss of HK expression leads to intrauterine fetal death. Also, a low prevalence of disease could indicate many HK deficiencies are incompatible with life.^{40,41,42} Mild cases may go undiagnosed because few laboratories are capable of testing for the mutation and due to the complicated laboratory and clinical diagnosis.⁴⁰

Individuals affected by HK deficiencies can have many associated ailments due to the mutations, including multiple malformations and psychomotor retardation.⁴² However, the predominating hallmark of the disease is a lifelong chronic nonspherocytic hemolytic anemia (HNSHA) that can range from mild to severe.⁴⁰

Glucose 6-Phosphate Isomerase Deficiency

Glucose 6-phosphate isomerase (PGI) is an enzyme that catalyzes glucose 6-phosphate and fructose 6-phosphate in the Embden-Meyerhof glycolytic pathway. This enzyme goes by several names, such as phosphoglucose isomerase and hexose phosphate isomerase, and has a chemical name of D-glucose-6-phosphate ketol isomerase. Previously, this enzyme was known as a housekeeping enzyme. Today, it is clear this enzyme is linked to an array of cytokine activities and extracellular processes.

A deficiency in GPI has been identified as a source of nonspherocytic hemolytic anemia since its discovery in 1968. This is the third most common form of inherited chemical deficiency. The GPI gene is located on chromosome 19. Over 40 causative mutations have been identified, resulting in GPI deficiency and an associated hemolytic anemia. Since its

discovery, over 50 different families have been documented to have GPI deficiency.³⁸ GPI is an autosomal recessive genetic disorder. GPI is considered a rare disease, and estimates of its presence in the population is unknown. Frequencies may be falsely represented due to the limited number of laboratories around the world that perform GPI enzyme assays. While rare, research demonstrates that more cases are likely to be discovered with the advent of advanced sequencing technologies that enable multiple gene characterization of similar hemolytic anemias simultaneously.^{38,39} The homozygous state of the null mutation for GPI that is governed by a single locus is lethal. Glycolysis and the pentose-phosphate shunt are required for erythrocyte metabolism. A homozygous null mutation results in complete GPI deficiency, which is lethal for the embryo. For this reason, live humans have not been studied with the disease state.

Biochemical testing for variants of this GPI mutation can prove difficult. Kanno and colleagues describe the instability of the GPI variant enzymes, but kinetics did not always appear to be impaired, making it difficult to identify variants by biochemical testing. When GPI deficiency is suspected, DNA analysis is required for diagnosis. This has also made it challenging to determine the phenotypic effect for each mutation.

Laboratory tests are available to assay many of the specific enzymes. Some of these tests may be available only through reference laboratories. Most laboratories, however, will be able to screen patients with a suspected hemolytic anemia caused by enzyme deficiency. The antiglobulin, erythrocyte survival, autohemolysis, osmotic fragility, and Heinz body tests can all be useful in distinguishing the enzyme deficiencies from hereditary spherocytosis and the unstable hemoglobinopathies.

Enzyme Deficiencies: Methemoglobin Reductase Pathway

Methemoglobin Reductase Deficiency

The **methemoglobin (MetHgb)** mutation was discovered in the 1960s by a young hematologist who traveled to a remote area in Appalachian Kentucky to study a family, famous for their blue skin.⁴⁴ The family had seven children, four of whom were carriers of the trait for methemoglobinemia. Due to the isolation, children in the family chose interfamily marriages, perpetuating the carriage of the mutation through the familial line.⁴⁴

Methemoglobin (MetHgb) is formed when hemoglobin is deoxygenated due to a superoxide or peroxide group. The iron in the heme group is oxidized from a ferrous state (Fe^{2+}) to a ferric state (Fe^{3+}). Heme with a ferric iron is unable to bind to oxygen, preventing erythrocytes from transporting oxygen throughout the body.

Under normal conditions, the peripheral blood contains <1% methemoglobin.²¹ The body has enzymes present to keep methemoglobin under 1%. The enzymes are cytochrome B5 reductase, biliverdin reductase B, nicotinic amide adenine dinucleotide phosphate (NADPH)-Methemoglobin reductase, and NADPH-Met-Hgb-diaphorase. These enzymes convert MetHgb back into hemoglobin with a ferrous iron.²¹

Methemoglobinemia

Methemoglobinemia is an increase in methemoglobin in the blood.

Pathogenesis and Clinical Findings

Methemoglobinemia can be acquired or hereditary. It can be caused by drugs or by the consumption of certain foods. Methemoglobinemia can also be inherited from a rare mutation found on the CYB5R3 gene as an autosomal recessive trait.⁴⁵ Individuals with this gene are deficient in CYB5R. Two varying types exist. Type I mutation occurs only in erythrocytes and type II mutation occurs in all cells.⁴⁵

Methemoglobinemia type I is a functional anemia, but the affinity for oxygen is significantly decreased in the erythrocyte.

The major clinical feature of inherited methemoglobinemia is cyanosis. Because methemoglobin cannot carry oxygen, some patients exhibit symptoms similar to those of anemia, and some patients develop a compensatory mild polycythemia (see Chapter 19). In addition to the hereditary deficiency of NADH-methemoglobin reductase, methemoglobinemia may be caused by the hemoglobin M diseases (see Chapter 11). Complications that arise from the disease are primarily due to hypoxia. Tolerance to the percentage of MetHgb in peripheral circulation varies from one individual to the next. At 70% MetHgb, the blood appears to be chocolate brown in color.⁴⁶ In methemoglobinemia type I,

the course of this disorder is generally benign; however, some patients are treated only because they find their lifelong cyanosis to be a cosmetic hardship. Methemoglobinemia type II is accompanied by complications such as neurological impairment, mental impairment, and growth malformations.⁴⁴

In cases of severe cyanosis, methylene blue is administered intravenously to activate the NADH-methemoglobin reductase system. The active ingredient, methylene blue trihydrate (3,7-bis (dimethylamino) phenazinium chloride trihydrate), is considered a prodrug.⁴⁶ Converted into a colorless leucomethylene blue by flavin reductase in the erythrocyte, MetHgb is converted to hemoglobin; at the same time, leucomethylene blue is converted to methylene blue.⁴⁶ The methylene blue is recyclable for the next conversion. While new methylene blue is suitable to treat methemoglobinemia, complications can arise because of its ability to oxidize hemoglobin and induce methemoglobinemia. The drug also has several side effects such as nausea, vomiting, diarrhea, abdominal pain, oral dysesthesia, blue saliva, blue stool, blue urine, headache, mental confusion, dyspnea, excessive perspiration, rash with severe burning, necrosis, abscess, and ulceration.⁴⁶

Acquired methemoglobinemia is usually an acute disease state, can be life threatening, and may have other complications such as hemolytic anemia.⁴⁶ Acquired methemoglobinemia is often drug-induced by drugs such as nitroglycerine, dapsone, sulfonamides, primacine, phenytoin, phenacetin, and prilocaine.⁴⁶ Acquired methemoglobinemia can occur from the consumption of high nitrate containing foods, such as beets, spinach, carrots, boronchard, or consumption of pesticide- or fungicide-contaminated water.⁴⁶ Most cases of acquired methemoglobinemia is manageable. However, cases that are most severe are dependent on the organ damage that occurs from the hypoxic episode.

Laboratory Testing and Results

The laboratory differentiation of the types of methemoglobinemia is shown in Table 10-4. Methemoglobin has a maximum absorbance band at 630 nm. The addition of cyanide causes the band to disappear, and the change in absorbance is directly proportional to the concentration of methemoglobin.⁴⁶ Methemoglobin is increased to varying degrees in all three disorders. Enzyme activity is decreased only in hereditary NADH-methemoglobin reductase deficiency. Hemoglobin electrophoresis produces normal-appearing results in patients with methemoglobinemia except in the hemoglobin M diseases.

CRITICAL THINKING QUESTION

10-3 How can laboratory testing differentiate hereditary methemoglobinemia from the acquired forms?

TABLE 10-4 Laboratory Differentiation of Types of Methemoglobinemia

Methemoglobinemia Resulting From	Methemoglobin Level	Enzyme Activity	Hemoglobin Electrophoresis
Hereditary enzyme deficiency	Increased	Decreased	Normal
Toxic substance exposure	Increased	Normal	Normal
Hemoglobin M disease	Increased	Normal	Abnormal

SUMMARY CHART

- Hereditary nonspherocytic hemolytic anemia (HNSHA) encompasses a group of disorders associated with red blood cell (RBC) abnormalities.
- Glucose-6-phosphate dehydrogenase (G6PD) enzyme abnormalities are the most common cause of HNSHA.
- G6PD is an enzyme in the hexose monophosphate (or pentose phosphate) shunt pathway.
- G6PD enzyme variants are noted for their association with particular racial and ethnic backgrounds.
- Pyruvate kinase, an essential enzyme of the Embden-Meyerhof pathway, is the second most common enzyme abnormality associated with HNSHA.
- Clinical expression of G6PD deficiency is more often evident in men because it is a sex-linked (X chromosome) abnormality.
- Deficient G6PD activity results in reduced glutathione levels, which cause increased oxidative denaturation of hemoglobin and subsequent hemolysis.
- Patients with G6PD abnormalities are usually asymptomatic until exposed to conditions of lowered oxygen tension, certain chemicals or substances (including fava beans), and some medications.
- G6PD is diagnosed by clinical symptoms and laboratory determinations. Laboratory testing includes finding the presence of Heinz bodies in erythrocytes (not specific), electrophoresis of G6PD, and, where indicated, molecular genetic studies.
- PK deficiency, which was first identified in 1961, is associated with a diminished capacity to generate ATP, resulting in fragile red blood cells and a hemolytic anemia.
- Laboratory diagnosis of PK deficiency requires specific testing of PK activity. A fluorescent screening test is used.
- Other red cell enzyme deficiencies include methemoglobin reductase and glucose phosphate isomerase deficiencies. Almost any enzyme of the aerobic and anaerobic metabolic pathways has been implicated in HNSHA, although the levels of hemolysis are quite variable and hemolysis may be entirely absent.

CASE STUDY 10-1

A 26-year-old African American man was referred to the clinical laboratory for investigation of reported hemoglobinuria. The patient had recently been diagnosed as having infectious mononucleosis. The following laboratory data were obtained:

RBC	$3.7 \times 10^{12}/L$
Hgb	11.0 g/dL
Hct	32%
MCV	86.0 fL
MCHC	34.0%
WBC	$9.5 \times 10^9/L$
Differential	
Segmented neutrophils	40%
Bands	3%
Lymphocytes	48% (many atypical)
Monocytes	7%
Eosinophils	2%
Platelets	Adequate
Reticulocytes	14.5% (uncorrected)

The red blood cell (RBC) morphology was normochromic and normocytic. Polychromasia was noted. A slight poikilocytosis was also noted, with some red cells showing irregular protrusions. On further investigation, the antiglobulin test result was found to be negative. On the basis of the

antiglobulin test, the hemolytic process was considered not to be the result of an immune reaction. A normal hemoglobin electrophoresis was reported.

The hematologist suggested that the patient return in 30 days for testing for erythrocyte enzyme deficiency. At that time, the patient was found to have an adequate erythrocyte G6PD content. Spuriously elevated G6PD levels may be found during or immediately after the hemolytic episode because of the presence of younger red blood cells.

QUESTIONS

1. Do the CBC results indicate hemolytic anemia?
2. What does the reticulocyte count in this patient represent?
3. What is a likely diagnosis for this patient?
4. What RBC inclusion might also be found on the peripheral blood smear with supravital stains?
5. What further testing can be performed in diagnosing G6PD deficiency?
6. What type of hemolysis is present here?

ANSWERS

1. Yes, the patient has a low RBC count, low Hgb and Hct, and high reticulocyte count. These results indicate low red blood cell population with increased erythropoietic turnover through bone marrow compensation.

Continued

CASE STUDY 10-1—cont'd

2. The reticulocyte count in this patient indicates that the bone marrow is attempting to compensate for the decreased red blood cell population by increasing the rate at which immature red cells are entered into the peripheral circulation.
3. G6PD deficiency is the most likely diagnosis based on pathogenesis of the condition and the patient's symptoms and genetic heritage.
4. Heinz bodies.
5. Bilirubin testing, haptoglobin levels, and lactate dehydrogenase.
6. G6PD definitely results in intravascular hemolysis.

REVIEW QUESTIONS

1. What is the most common glycolytic enzyme deficiency associated with the pentose phosphate pathway (aerobic pathway)?
 - a. PK deficiency
 - b. G6PD deficiency
 - c. Hexokinase deficiency
 - d. Glutathione reductase deficiency
2. What is the most common glycolytic enzyme deficiency associated with the Embden–Meyerhof pathway (anaerobic pathway)?
 - a. PK deficiency
 - b. G6PD deficiency
 - c. Hexokinase deficiency
 - d. Glutathione reductase deficiency
3. Oxidative denaturation of hemoglobin results in formation of small particles that are visualized with supravital staining, referred to as:
 - a. Basophilic stippling
 - b. Howell–Jolly bodies
 - c. Pappenheimer bodies
 - d. Heinz bodies
4. In the evaluation of a patient for G6PD deficiency, which of the following test results would indicate a deficiency of the enzyme?
 - a. Increased formation of schistocytes
 - b. Increased fluorescence in the fluorescent spot test
 - c. The reduction of methemoglobin in the presence of methylene blue
 - d. An abnormal autohemolysis test
5. Which laboratory test result would indicate a patient who is PK-deficient?
 - a. Abnormal rate of hemolysis that is independent of the presence or absence of glucose in the incubation medium of the autohemolysis test
 - b. Lack of fluorescence in the fluorescent spot test
 - c. A change in the indicator from red to yellow in the orthocresol red test
 - d. Increase in osmotic fragility
6. What deficiency causes hemoglobin to be oxidized from the ferrous to the ferric state?
 - a. G6PD deficiency
 - b. PK deficiency
 - c. NADH-methemoglobin reductase deficiency
 - d. Lactate dehydrogenase deficiency
7. G6PD deficiency will cause which of the following clinical findings?
 - a. Positive DAT
 - b. Spherocytes
 - c. Acute hemolysis
 - d. Increased hemoglobin
8. Which stain is required to visualize Heinz bodies?
 - a. Romanowsky stain
 - b. Crystal violet
 - c. Wright's stain
 - d. Gram stain
9. A major difference between Pk deficiency and hemoglobinopathy is:
 - a. Spherocytes
 - b. Heinz bodies
 - c. Helmet cells
 - d. No abnormal RBC morphologies present
10. What is a key peripheral finding in PK deficiency?
 - a. Schistocytes
 - b. Spherocytes
 - c. nRBCs
 - d. Heinz bodies
11. What do all glycolytic enzyme deficiencies have in common?
 - a. Increased retic counts
 - b. Intravascular hemolysis
 - c. Chronic nonspherocytic hemolytic anemia
 - d. Only mild cases seen
12. Which of the following is true about acquired methemoglobinemia?
 - a. Often an acute, but manageable condition
 - b. Causes severe cyanosis
 - c. Causes neurological and mental impairment
 - d. Causes growth malformations

See answers at the back of this book.

Hemolytic Anemias

Intracorpuscular Defects: The Hemoglobinopathies

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CHAPTER OUTLINE

Introduction	Laboratory Screening for Sickle Cell Disease	Hemoglobin S/ β -Thalassemia Combination
Review of Normal Hemoglobin Structure	Treatment	Laboratory Diagnosis of HbS With Other Abnormal Hemoglobins
Overview of the Hemoglobinopathies	Hemoglobin C Disease and Trait	Hemoglobin Variants With Altered Oxygen Affinity
Classification	Hemoglobin D Disease and Trait	Unstable Hemoglobins
Nomenclature	Hemoglobin E Disease and Trait	Methemoglobinemia
Sickle Cell Anemia	Hemoglobin O _{Arab} Disease and Trait	Overview of Laboratory Diagnosis of Hemoglobinopathies
Historic Overview	Hemoglobin S With Other Abnormal Hemoglobins	Case Study 11-1
Definition	Hemoglobin SC Disease	Summary Chart
Pathophysiology	Hemoglobin SD Disease	Review Questions
Clinical Findings	Hemoglobin SO _{Arab} and S-Oman Disease	
Sickle Cell Trait		
Laboratory Testing and Results		

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- | | |
|---|---|
| <p>11-1 Describe the structure and molecular composition of normal hemoglobin.</p> <p>11-2 Name the correct state of iron required for proper delivery and transport of oxygen.</p> <p>11-3 Identify the most common globin chain abnormality-causing hemoglobinopathies.</p> <p>11-4 Differentiate qualitative hemoglobin defects from quantitative hemoglobin defects.</p> <p>11-5 Name the amino acid substitution found in sickle cell anemia.</p> <p>11-6 List factors contributing to the sickling process.</p> <p>11-7 Describe the clinical findings, including hallmark symptoms, of sickle cell anemia.</p> | <p>11-8 Describe the goals of treatment for sickle cell anemia.</p> <p>11-9 Describe the relationship between infections, including parasitic infections and sickle cell anemia.</p> <p>11-10 List the appropriate laboratory testing and expected results for sickle cell anemia.</p> <p>11-11 Name the amino acid substitutions found in HbC and HbD diseases.</p> <p>11-12 List the laboratory findings in HbC and HbD diseases.</p> <p>11-13 Describe the predominant cellular abnormalities seen in HbE and Hb O_{Arab} diseases and traits.</p> <p>11-14 Identify the laboratory findings that indicate a diagnosis of hemoglobin SC disease.</p> <p>11-15 Describe the characteristics of unstable hemoglobins.</p> <p>11-16 Identify the causes of methemoglobinemia.</p> |
|---|---|

Hemoglobinopathies are defined in the broadest sense as conditions in which there are either qualitative or quantitative abnormalities in the synthesis of hemoglobin. More than 1,000 hemoglobin variants with a single amino acid substitution have been discovered.¹ However, the majority of these variants are not clinically significant, because the abnormality does not result in a defect in the structural integrity or function

of the hemoglobin molecule.² The hemoglobinopathies are either inherited according to classic mendelian genetics or arise from new genetic mutations.

More than 90% of the hemoglobin variants are single amino acid substitutions in the alpha (α), beta (β), delta (δ), or gamma (γ) globin chains as a result of a single-point mutation in one of the globin genes. Hemoglobinopathies are inherited

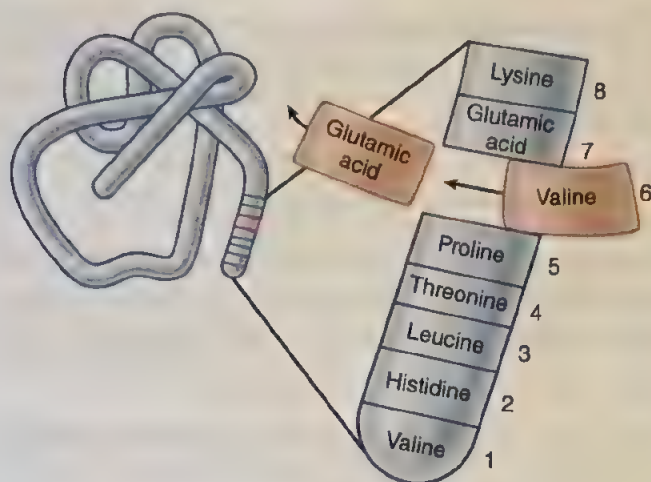


FIGURE 11-3 Amino acid substitution in hemoglobin S.

factors are responsible for the appearance of this hemoglobin in North American and Middle Eastern populations.³ In the United States, the birth incidence of the homozygous state (HbSS) is approximately 0.26% (1 in 375 African American babies).^{3,9} It is estimated that 1 in 12 African Americans (8%) carry the autosomal recessive mutation trait (one gene) for HbS. Approximately 300,000 infants are born with sickle cell anemia annually.¹⁰

Although in the United States sickle cell disease is most commonly found in persons of African ancestry, it has also been found in individuals from the Caribbean, South and Central America, Mediterranean (Turkey, Greece), the Middle East, and India.¹¹ The common variants of sickle cell disease are listed in Table 11-2, with their estimated incidence.²

Pathophysiology

HbS is soluble and usually causes no problem when properly oxygenated. However, when the oxygen tension decreases, this single amino acid substitution in the β -globin chain of HbS polymerizes, forming tactoids or fluid polymers (Fig. 11-4).¹² As these polymers realign, they cause the red cell to deform into the characteristic sickle shape (Fig. 11-5). The sickling process is dependent on the degree of oxygenation, pH, and dehydration of the patient.¹¹ Decreases in oxygenation and pH, as well as dehydration, promote sickling.

There are two types of sickled cells: reversible and irreversible.¹¹ Sickling of the red cell is reversible up to a point. However, repeated sickling eventually damages the red blood cell (RBC) membrane permanently. Originally, the formation of rigid sickled cells was thought to cause microcirculatory



FIGURE 11-4 Scanning electron micrograph (SEM) of sickle cells. (From Bell A. Hematology. In: Listen, Look and Learn. Bethesda, MD: Health Education Resources, Inc. with permission.)

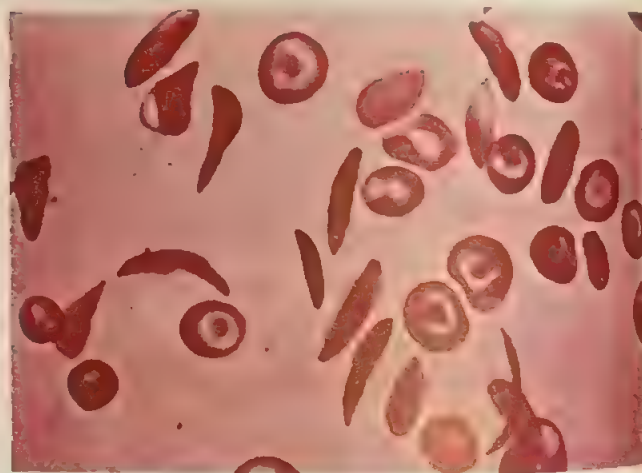


FIGURE 11-5 Sickle cell disease (peripheral blood). Note the sickle-shaped red cells and target cells. (From Bell A. Hematology. In: Listen, Look and Learn. Bethesda, MD: Health Education Resources, Inc. with permission.)

obstruction because of impaired erythrocyte deformability during capillary transit. However, the actual mechanism involves several other elements, accounting for the phenotypic heterogeneity of this disease. These mechanisms involve: 1. the adhesion of erythrocytes to the endothelium of the post-capillary venule; 2. the formation of aggregates composed of irreversibly sickled cells and leukocytes contributing to vascular occlusion and inflammation; 3. neutrophil transmigration through vessel walls adding to further increased inflammation in the microvasculature; and 4. dysregulation of vasomotor tone by disturbance in the function of vasodilator mediators such as nitric oxide.¹² In addition, abnormal cation homeostasis caused by hemoglobin S polymers leads to sickle red cell dehydration, producing dehydrated, dense, irreversibly sickled cells that not only result in hemolytic anemia but also play a crucial role in the initiation of vaso-occlusion.¹² Vaso-occlusion then further lowers pH and oxygen tension and increases the numbers of sickled cells, resulting in both acute and chronic tissue damage.¹³ This injury results in painful crises and the infarction of organs. It should be noted that the presence of hemoglobin F (HbF) and HbA₂ in red cells with HbS modifies the degree of severity of the sickling.^{3,9-12} Other factors affecting the severity of HbS are listed in Box 11-1.

TABLE 11-2 Sickle Cell Disease (a Group of Genetic Disorders Characterized by the Production of HbS)

Disorder	Incidence in African American Live Births
Sickle cell anemia (HbSS)	1 in 375 (0.26%)
Sickle cell C disease (HbSC)	1 in 835 (0.12%)
Sickle thalassemia	1 in 1,667 (0.06%)

Source: Sickle Cell Disease Guideline Panel. Sickle cell disease: Screening, diagnosis, management, and counseling in newborns and infants. Clinical Practice Guideline No. 6. AHCPR Pub No 93-0562. Rockville, MD: U.S. Department of Health and Human Services; April 1993.

BOX 11-1 Factors Affecting the Severity of HbS

- Amount of HbS
- Vascular stasis
- Other hemoglobins
- Temperature
- Thalassemia
- pH
- Glucose-6-phosphate dehydrogenase (G6PD) deficiency
- Viscosity
- Deoxygenation
- Mean corpuscular hemoglobin concentration
- Amount of HbF
- Dehydration

Clinical Findings

The hallmark features of sickle cell disease are chronic hemolytic anemia and vaso-occlusion, resulting in ischemic tissue injury.¹⁴ All tissues and organs within the body are at risk for damage because of the vascular obstruction produced by the sickled red cells. Organs at greatest risk include the spleen, kidney, and bone marrow because, in these organs, blood flow is slow in the venous sinuses and there is a reduced oxygen tension and low pH.¹³ The eye and head of the femur are also target sites for ischemic injury because of the limited terminal arterial blood supply to these areas.

Sickle cell anemia is usually diagnosed early in life when the level of HbF declines. HbSS disease typically presents as a severe chronic hemolytic anemia, with hemoglobin levels in the range of 6 to 8 g/dL.¹⁴ Characteristically, the patient demonstrates an asthenic physique and is mildly jaundiced (Fig. 11-6). Many complications are associated with the disease, with the major manifestations being "sickle crises." There are three types of crises: aplastic, hemolytic, and painful (vaso-occlusive).^{14,15}

An aplastic crisis is usually associated with infections, particularly to parvoviruses, which cause a temporary suppression of erythropoiesis. The marrow is simply overworked as a result of the stress related to the continuous stimulus for production of new red cells. With an already shortened red cell life span, even a temporary decrease or arrest in red cell production causes a drastic anemia. During the evaluation of the febrile patient, a fall in the reticulocyte count can also indicate the onset of aplastic crisis, which requires further monitoring of the hemoglobin level in the HbSS disease patient. Aplastic crises usually spontaneously resolve within 5 to 10 days.¹⁵

A hemolytic crisis reflects an acute exacerbation of the anemia with a resulting fall in hemoglobin and hematocrit, an increased reticulocyte count, and jaundice.^{14,15} Acute splenic sequestration is the cause, resulting in a decrease in hemoglobin and hematocrit, which usually occurs in infants and young children between 5 months and 2 years of age.¹⁵ Intrasplenic pooling of vast amounts of blood results in enlarged spleens of some children with HbSS disease. The usual clinical features of a hemolytic crisis include sudden weakness, rapid pulse, faintness, pallor of the lips and mucous membranes, and abdominal fullness caused by the enlarged spleen.¹⁵ In

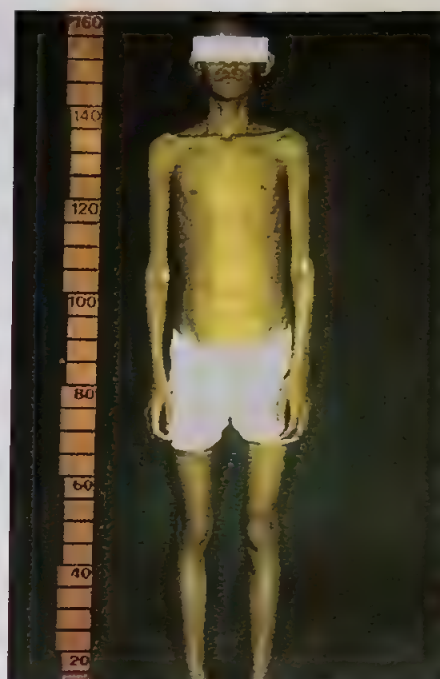


FIGURE 11-6 Asthenic physique with mild jaundice. (Reproduced with permission from Sandoz Pharmaceuticals Corporation.)

contrast to this process, multiple infarctions and subsequent fibrosis lead to a process termed *autosplenectomy* in adult patients with HbSS disease, which results in a small, fibrotic, and nonfunctional spleen.¹⁵

Vaso-occlusive or painful crisis is the hallmark of sickle cell anemia.¹⁵ The crisis is usually associated with severe pain, caused by occlusion of small blood vessels mediated by the adhesion of sickled cells to endothelium, resulting in tissue damage and necrosis.¹⁵ The decreased blood flow causes regional hypoxia and acidosis, further exacerbating the ischemic injury. A painful crisis usually lasts 4 to 6 days but sometimes persists for weeks.⁹ Painful crisis can be precipitated by infection, fever, acidosis, dehydration, and exposure to extreme cold.⁹ Some patients have reported that even emotional states such as anxiety, stress, and depression may cause their painful crises.⁹

Generally, three principles of therapy are applied in the management of painful crisis:

1. Adequate rehydration
2. Pain relief using sufficient analgesics
3. Antibiotic therapy to treat any precipitating or underlying illness such as infection^{15,16}

In severe cases, transfusion may be necessary to reduce the hemoglobin S content in the blood of the patients with HbSS disease.¹⁷ However, the mainstay of therapy for painful crises is hydration (administration of fluid volumes) to correct fluid and electrolyte deficits in an attempt to maintain normal serum electrolyte concentrations.^{16,17}

Symptoms and clinical manifestations of HbSS disease are many and varied. The clinical presentations of sickle cell anemia represent the sequelae of repeated infarction. The common clinical findings are listed in Table 11-3 by hematologic and nonhematologic categories.

TABLE 11-3 Clinical Features of Sickle Cell Anemia by Category

Category	Clinical Features
Hematologic	<ul style="list-style-type: none"> • Aplastic crisis • Hemolytic crisis • Vaso-occlusive crisis
Nonhematologic	<ul style="list-style-type: none"> • Abnormal growth • Bone and joint abnormalities <ul style="list-style-type: none"> • Arthritis • Pain • <i>Salmonella</i> infection • Hand-foot dactylitis • Bone marrow infarctions • Genitourinary <ul style="list-style-type: none"> • Gastrointestinal symptoms • Renal papillary necrosis • Priapism • Spleen and liver <ul style="list-style-type: none"> • Autosplenectomy • Hepatomegaly • Jaundice • Cholelithiasis • Cardiopulmonary <ul style="list-style-type: none"> • Enlarged heart • Heart murmurs • Pulmonary infarction • Eye <ul style="list-style-type: none"> • Retinal hemorrhage • Conjunctival vascular abnormalities • Central nervous system • Leg ulcers • Risky pregnancy

Vasculopathy

Occlusion of blood vessels and tissue ischemia can occur virtually anywhere in the body; in bones, joints, lungs, liver, kidneys, eye, central nervous system, and spleen.^{8,9} In the lungs, sickling in the pulmonary microvasculature produces the acute chest syndrome in HbSS disease patients. It is the second most common cause of hospital admission and the most common complication of surgery and anesthesia.^{16, 18} The acute chest syndrome represents an acute illness characterized by fever, chest pain, prostration, and the presence of pulmonary infiltrates on the chest x-ray exam.^{14,16} The syndrome in adults is characteristically a result of pulmonary infarction, although other causes such as bacterial or viral infection have been reported. This contrasts with the acute chest syndrome in children with HbSS disease, which is usually caused by an infectious agent. While children have a higher incidence of acute chest syndrome, their mortality rate is lower than that of adults.¹⁷ Pleuritic chest pain is the dominant symptom of acute chest syndrome in adults, whereas fever, cough, and tachypnea are often the only complaints in infants and young children who are affected.^{14,16}

The most common cutaneous manifestation in HbSS disease is the development of ulcers or sores on the lower leg (Fig. 11-7). Approximately 8% to 10% of patients develop leg



FIGURE 11-7 Leg ulcers in a patient with sickle cell anemia. (Reproduced with permission from Sandoz Pharmaceuticals Corporation.)

ulcers, which are usually manifested between 10 and 50 years of age and are very difficult to resolve.^{14,16,19} The etiology of leg ulcers is unclear; however, trauma, infection, severe anemia, and warmer temperatures may predispose to their formation.¹⁹ They rarely develop in individuals with sickle cell C disease (HbSC) or sickle β thalassemia.²

Bones and joints in HbSS disease are frequent sites of pathology, with musculoskeletal pain being the most common symptom. There can be bone marrow hyperplasia, infection, or infarction. Infarction is also commonly responsible for the symptoms of the hand-foot syndrome observed in sickle cell anemia. **Dactylitis** (the painful swelling of the hands and feet) occurs commonly in infants and young children with HbSS disease and is observed exclusively in patients in that age group.^{15,16,19} In many infants it is the first manifestation of the disease. The characteristic "hand-foot" syndrome develops later in life because of microinfarction of small bones of the hands and feet, which leads to unequal growth and bone deformities of the fingers and toes (Fig. 11-8). In addition, episodes of painful swollen joints and aseptic necrosis of the femoral head and other articulating bones are caused by the process of infarction.

Infections

Serious bacterial infections remain a major cause of morbidity and mortality in patients with HbSS disease. The organisms implicated in causing infections in these patients are listed in Table 11-4.

The most significant cause of death during early childhood is the severe overwhelming septicemia and meningitis caused by *Streptococcus pneumoniae*.^{1,3,16,18} In HbSS disease, splenic dysfunction develops during infancy and predisposes the infant to overwhelming infections from encapsulated



FIGURE 11-8 Hand-foot syndrome in a patient with sickle cell anemia. (Reproduced with permission from Sandoz Pharmaceuticals Corporation.)

bacteria, such as *S. pneumoniae* and *Haemophilus influenzae*.^{8,9,14} After the first decade of life, anaerobic and enteric organisms become important pathogens, causing infections in adult patients with HbSS disease. Repeated splenic infarcts result in autosplenectomy by the adult years. These patients then become more prone to serious infections with encapsulated organisms. Infections in patients with HbSS disease cause greater morbidity, disseminate more rapidly,

and are more difficult to resolve than infections in healthy individuals. In particular, pyelonephritis recurs regularly in these patients, is difficult to treat, and is often associated with septicemia.^{1,3,18} This infection results in a predisposition to sickling in the renal papilla, ultimately causing renal papillary necrosis, which frequently develops along with the pyelonephritis.

There are multiple factors responsible for the increased susceptibility to infection in patients with HbSS disease, including^{3,11,15}:

- Reticuloendothelial blockage caused by increased hemolysis
- Stasis of sickled RBCs in the sinusoids of the liver and spleen
- Secondary splenic dysfunction
- Deficiency of nonantibody serum opsonic activity

The relationship between the incidence of the malarial parasite and the frequency of the abnormal HbS gene requires further explanation. Malaria, caused by a parasite of the *Plasmodium* species, is still a serious disease in tropical areas, with *Plasmodium falciparum* being responsible for the most life-threatening situations. The original geographic distribution of sickle cell disease overlaps with that of malaria. As in thalassemias, these hemoglobinopathies are believed to provide some kind of selective advantage for malaria. This is applicable only to subjects carrying one abnormal HbS gene (sickle cell trait). The precise mechanism of this protective effect from malaria is not known; however, cells carrying HbS, when parasitized by *P. falciparum*, may sickle more quickly than nonparasitized cells, leading to preferential destruction of the parasitized cells.¹⁸

The sickling could affect the cycle of the parasite in one of two ways: directly, by killing the parasite, or indirectly, by causing the parasitized sickle cells to be sequestered in the spleen. The fact that persons homozygous for the HbS gene often lack splenic function by the time they reach adulthood (autosplenectomy or functional asplenia) may be one reason why malaria is exceptionally severe, and often fatal, in these cases.¹⁸ Thus, HbS confers a relative degree of protection from malaria only in the heterozygous (trait) state. It is

TABLE 11-4 Organisms Implicated in Causing Infections in Patients With Sickle Cell Anemia

Bacterial	Viral	Fungal	Parasitic
<i>Streptococcus pneumoniae</i>	Rubeola	<i>Coccidioides immitis</i>	<i>Plasmodium</i> species
<i>Haemophilus influenzae</i>	Cytomegalovirus	<i>Histoplasma capsulatum</i>	
<i>Neisseria meningitidis</i>			
<i>Mycoplasma pneumoniae</i>			
<i>Staphylococcus aureus</i>			
<i>Streptococcus pyogenes</i>			
<i>Mycobacterium tuberculosis</i>			
<i>Escherichia coli</i>			
<i>Salmonella</i> species			

balanced by the decreased fitness of the homozygous state, and the gene frequency eventually reaches an equilibrium in the population.

Sickle Cell Nephropathies²⁰

In the kidney, intravascular sickling occurs more rapidly than in any other organ owing to deoxygenation of HbS in the acidic and hyperosmolar environment of this organ.²⁰ The combination of hypoxia, hypertonicity, and acidosis in the kidney causes sickling, stasis, and ischemia of the renal medulla and papillary tip, leading to progressive renal events.²⁰ Eventually, over time, several sickle cell nephropathies develop.

Hyposthenuria, the inability of the kidney to concentrate the urine, is the earliest and most common nephropathy in sickle cell disease, occurring usually in the first decade.²⁰ Progressive renal pathology occurs in patients with HbSS disease as renal tubular dysfunction and atrophy presents itself in the second decade of life. The third decade in HbSS disease is characterized by interstitial nephritis, papillary necrosis, pyelonephritis, and the nephrotic syndrome, to name a few of the renal disorders that may develop. In the fourth decade and beyond, end-stage renal disease develops as one or more of the sickle cell nephropathies results in chronic renal failure.²⁰

It should be noted that hyperuricemia and gross hematuria occur commonly in patients with HbSS disease. Hyperuricemia occurs in approximately 15% of children and 40% of adults with HbSS disease because of the increased urate production associated with the accelerated erythropoietic rate and decreased renal clearance of urate.²⁰ Gross hematuria occurs commonly not only in sickle cell anemia but also in sickle cell trait because of the sickling, stasis, ischemia, and extravasation of blood in the kidney.²⁰

Stroke

Stroke occurs in up to 22% of patients with HbSS disease.^{11,21} Stroke represents an array of neurological complications caused by an ischemic or hemorrhagic lesion in a specific cerebral vessel. The neurological manifestations may be focal, such as hemiparesis, or more generalized, such as coma or seizure.²¹ Recurrent episodes of stroke cause progressively greater impairment and increased mortality. The most common cause of stroke in children is cerebral infarction.²¹ With age, subarachnoid and intracerebral hemorrhage become increasingly common.

A distinguishing pathological feature of arterial vessels involved in stroke is the presence of intimal and medial proliferation, which results in severe restriction of blood flow and is found in approximately 80% of angiograms of patients with sickle cell anemia and stroke.²⁰ Most likely this is owing to the interaction of sickled cells with endothelium, which results in endothelial damage, inflammation, and the local release of growth factors that stimulate proliferation of subintimal and medial cells. In the later stages of this vasculopathy, chronic ischemia leads to the formation of a delicate subcortical network of collateral vessels that are prone to rupture, likely accounting for the increased incidence of hemorrhagic stroke with increasing age.²⁰ HbSS disease patients with hemorrhagic stroke (intracerebral or subarachnoid hemorrhage) have a high mortality rate during the acute stage (may be as high as 50%).²⁰

Patients with a characteristic abnormality of cerebral blood flow (higher than normal velocity of flow on transcranial Doppler ultrasonography) have been shown to be at much greater risk to develop stroke.²⁰

Sickle Cell Trait

In sickle cell trait (Fig. 11-9), the heterozygous form of the disease, individuals inherit both a normal β -globin gene and a sickle globin gene (β^S). As a result, individuals with sickle cell trait produce both normal HbA and HbS, with a predominance of HbA in an approximate ratio of 60:40.²² The structural formula is $\alpha_2\beta_1\beta_1^{6Glu-Val}$. The frequency of this heterozygous condition in American blacks is approximately 8%.²² In vitro experiments demonstrate cells that are homozygous for hemoglobin S sickle when the oxygen level is decreased to 4% to 6%, whereas cells heterozygous for hemoglobin S (sickle cell trait) sickle when the oxygen level is decreased to 2%.²² Individuals with HbS trait are usually asymptomatic, but occasionally episodes of hematuria and hyposthenuria occur because of sickling in the kidney. The potential for sickling exists, therefore, and drastic lowering of pH or reduction in oxygen tension can precipitate a crisis. Causes for these include severe respiratory infections, air travel in unpressurized aircraft, anesthesia, and congestive heart failure. Even excessive exercise can lead to a significant buildup of lactic acid, resulting in sickling and subsequent infarction. Several deaths of American black soldiers with sickle cell trait have been reported as a result of rigorous basic training at altitudes greater than 4,000 feet, which led to a buildup of lactic acid, followed by acidosis and subsequent organ infarction.²²

Laboratory Testing and Results

The following laboratory tests should be performed to diagnose sickle cell disease: the complete blood count, a reticulocyte count, evaluation of the peripheral smear, hemoglobin electrophoresis or isoelectric focusing (IEF), and measurement of hemoglobins A₂ and F by high-performance liquid chromatography (HPLC).^{6,14}

The chronic anemia of HbSS typically is quite severe, with hemoglobins ranging between 6 and 8 g/dL. The RBC indices are normochromic and normocytic. The peripheral

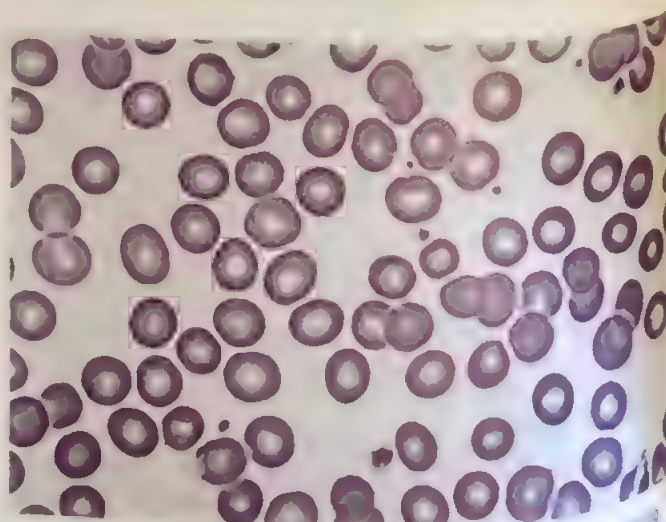


FIGURE 11-9 Sickle trait (peripheral blood). Note the normal appearance of the smear.

red blood picture can be striking, with numerous target cells, fragmented red cells, polychromasia, nucleated red cells, and usually sickled cells (see Fig. 11-5). Siderotic granules and Howell-Jolly bodies may be seen in the red cells as a result of rapid RBC turnover and "stressed" erythropoiesis. The average reticulocyte count is between 5% and 20%.⁶ This count decreases, however, during an aplastic crisis; indeed, a falling reticulocyte count may herald the onset of such a crisis. There may be a neutrophilic leukocytosis with a shift to the left and thrombocytosis. The bone marrow reflects marked erythroid hyperplasia, except during an aplastic crisis.

In individuals with the trait, sickled cells are not typically present on the peripheral blood smear. On rare occasions, however, sickled cells may be observed in the peripheral blood smear during a crisis episode.²²

Laboratory Screening for Sickle Cell Disease

According to the Clinical Practice Guideline on Sickle Cell Disease published by the U.S. Department of Health and Human Services, all newborns, regardless of race or ethnic background, should be screened for the presence of HbS.⁹

Newborn screening for sickle cell disease began in the United States in the early 1970s. The initial screening programs grew out of the recognition that sickle cell anemia was associated with significant morbidity and mortality. Today, newborn hemoglobinopathy screening is universally required by Law or Rule in the 50 U.S. states, the District of Columbia, Puerto Rico, and the Virgin Islands.²³

The majority of newborn screening (NBS) programs currently use HPLC and/or IEF to screen for SCD.^{23,24} Most programs retest abnormal screening specimens using a second, complementary electrophoretic technique, HPLC, immunological tests, or DNA-based assays.²⁴

Two previously used screening tests should **not** be used for newborn screening. These are (1) the sickle cell preparation using sodium metabisulfite and (2) solubility tests using a concentrated phosphate buffer, a hemolyzing agent, and sodium dithionite. These tube solubility tests either isolate HbS at an interface or cause the abnormal hemoglobin to precipitate (Fig. 11-10). Both tests depend on the concentration of HbS in the red cell or hemolysate. These tests are inappropriate screening tests because they are not sufficiently

sensitive to detect low levels of HbS and do not distinguish between sickle trait and different forms of sickle cell disease.

The predominance of HbF and low level of HbS in cells of the neonate is believed to explain the unreliability of these tests during the newborn period.

ADVANCED CONTENT

IEF is an equilibrium process in which hemoglobin migrates in a pH gradient to a position of 0 net charge. The formation of discrete, sharp bands via IEF allows for more precise and accurate quantification than standard electrophoresis.²⁴ HPLC is emerging as the method of choice for the initial screening of hemoglobin variants and quantification of HbA₂ and HbF. Although a greater number of samples can be processed daily using IEF, the advantages of HPLC are that it is generally easier to interpret; less prone to inconsistencies related to sample preparation, application, and staining techniques; and is fully automated, thereby reducing the chance for clerical error.²⁴ Mass spectrometry (MS) is emerging as a possible screening methodology with the introduction of robust and simple-to-use tandem MS instruments capable of greater throughput with comparable sensitivity and specificity of IEF and HPLC, in preliminary studies.²⁵ Molecular screening and clinical genotyping for hemoglobinopathies using rapid targeted next-generation sequencing platform has been evolving for accurate detection.^{26,27}

In the clinical laboratory, hemoglobin electrophoresis on cellulose acetate at alkaline pH, followed, if necessary, by electrophoresis on citrate agar at acid pH is performed to detect HbS²⁸ (Fig. 11-11). Electrophoresis separates different hemoglobins by electrical charge, pH, and different media. Hemoglobin separation by citrate agar electrophoresis depends on the combination of charge and the ability of the hemoglobin to combine with components in the agar gel mixture. The patient with sickle cell anemia produces no normal β chains; therefore, there will be no HbA on electrophoresis (unless the patient has been recently transfused). HbS constitutes 80% or more of hemoglobin, with HbF ranging from 1% to 20%.²⁹ When HbF levels are higher than 20%, there is a decrease in the severity of the disease.^{1,9,29} High levels of HbF

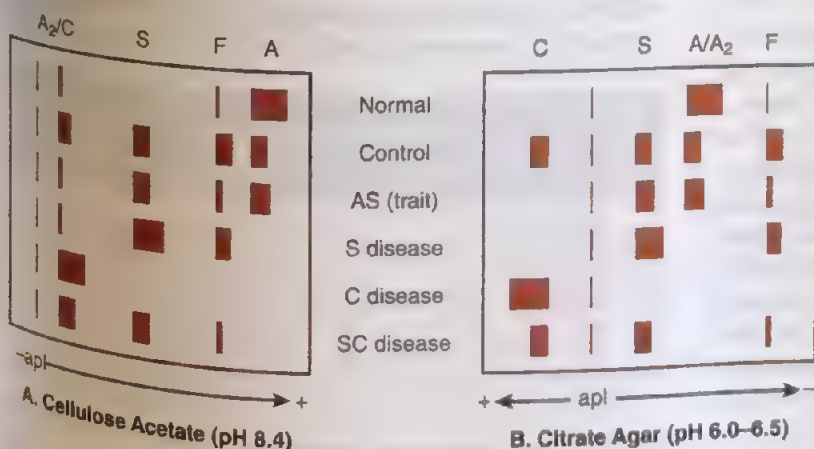


FIGURE 11-10 Electrophoretic patterns of hemoglobin on (A) cellulose acetate, run at pH 8.4, and (B) citrate agar, run at pH 6.0 to 6.5. apl = point of application.



FIGURE 11-11 Tube solubility screening test for sickle cell anemia.

are seen transiently in newborns and in hereditary persistence of fetal hemoglobin (HPFH).²⁹ In sickle cell anemia, the HbA₂ level may be slightly increased, with a mean of 3.4%.^{1,9,24} Hemoglobins with similar charges have similar migration patterns during electrophoresis, especially on cellulose acetate. Hemoglobins D and G both migrate to the same position as HbS at alkaline pH. Hemoglobin E (HbE) and hemoglobin O_{Ara} (HbO_{Ara}) migrate in the same position as HbC.²⁸ Citrate agar electrophoresis is very useful, because it clearly separates HbS from HbG and HbD, and HbC from HbE and HbO_{Ara} at acid pH.²⁸ In sickle cell trait, hemoglobin electrophoresis at alkaline pH shows 60% HbA, 40% HbS, and usually elevated HbA₂ (mean is 3.6%).²⁸ At acid pH, one band is present in the A position (HbA + HbA₂), whereas the other band migrates to the S position (see Fig. 11-10).²⁸

ADVANCED CONTENT

It should be noted that most hemoglobin variant traits, without coexistent conditions such as iron deficiency or thalassemia, have alkaline electrophoretic patterns with an approximate 60:40 ratio of normal to abnormal hemoglobin. This is because of the effect of charge on assembly of globin chains: relatively positively charged globins, such as S (+1) and C (+2), have a slight disadvantage in assembling with the α chains to form $\alpha\beta$ dimers compared with normal β globin. For this reason, HbS comprises less than 50% of the hemoglobin in heterozygotes, and a further reduction is seen in HbC trait.

Although sickle cell disease should not be diagnosed from either a sickle cell preparation or solubility test, because neither of these tests will reliably distinguish sickle cell trait from sickle cell anemia, many hospitals still perform these tests on adult patients. It is important to note that some rare hemoglobins also sickle, giving positive solubility tests. Box 11-2 lists examples of rare hemoglobins that sickle and give a positive tube solubility test. Because HbS, HbG, and HbD all migrate in the same position on cellulose acetate electrophoresis, it is helpful to know that HbG and HbD do not give a positive tube solubility test.

Molecular diagnosis of hemoglobin disorders using automated DNA-based techniques is available, but these techniques remain expensive and are most effective when targeted to a specific mutation.²⁷

Next-generation sequencing (NGS) is a technology that can be applied to the whole genome, the exome, or targeted gene panels. This technology has demonstrated a rapid, multiplex, and high throughput in detecting genetic variants.²⁷

ADVANCED CONTENT

Preimplantation genetic testing (PGT) is available and mainly utilized for couples at high risk of transmitting an inherited genetic disorder to their offspring.²⁷ The most common reason to perform a PGT is to avoid the conditions associated with hemoglobin synthesis caused by mutations in the beta-globin gene. The demand for PGT is increasing but the challenge is the vast diversity of potentially affected genotypes that involves creating individual protocols and customization to detect a specific combination of mutations.²⁷

Treatment

With advances in the diagnosis, treatment, and prevention of complications, the life expectancy of individuals with sickle cell disease has improved. Infants born in the United States with HbSS disease now have a life expectancy of 30 to 50 years old.³⁰ Delay in diagnosis and treatment resulting from lack of appropriate health services plays an important role in overall morbidity and mortality in developing countries.

In the past, the principal causes of death in infants with HbSS disease in the United States was overwhelming infections with *S. pneumoniae*, cerebrovascular accidents, and acute

BOX 11-2 Examples of Rare Hemoglobins That Sickle and Give a Positive Tube Solubility Test

- HbC_{Harlem}
- HbC_{Georgetown}
- HbC_{Ziguinchor}
- HbS_{Memphis}
- HbS_{Travis}
- Hb_{Alexandria}
- Hb_{Porto-Alegre}

splenic sequestration crises.³¹ One of the greatest advances in therapy of sickle cell disease has been the introduction of prophylactic penicillin therapy, which has virtually eliminated pneumococcal sepsis, one of the major causes of death in children with sickle cell disease.³¹ Twice-daily administration of oral penicillin reduces both morbidity and mortality from pneumococcal infection in HbSS disease in infants.³¹ In addition, administration of age-appropriate immunizations should be given, including pneumococcal, conjugated *H. influenzae*, and hepatitis B vaccines.¹⁴

A variety of drugs are being tested for their potential in ameliorating the effects of sickle cell anemia. Development of drug therapies is based on the pathophysiology of sickle cell disease.^{14,16,19} Studies have shown that increasing HbF decreases HbS polymerization; this effect is based on the reduction in concentration of HbS and on a direct inhibition of polymerization by HbF. A number of candidate agents for increasing HbF have been evaluated in the past 25 to 30 years. Several studies in small groups of patients with HbSS disease showed an increase in HbF levels and an increase in the fraction of cells containing HbF (F cells) with hydroxyurea therapy.³² These findings were dramatically confirmed in the randomized, double-blind multicenter study of hydroxyurea in patients with sickle cell disease.³²

The goals of these previously researched therapeutic approaches for the treatment of sickle cell anemia are listed in Box 11-3. Various approaches to specific therapy with specific drugs are listed in Table 11-5.

Blood transfusions may be required for acute situations and for prevention of certain complications of HbSS disease, such as stroke.²¹ Simple transfusion has little or no benefit for treatment of acute sickle vasculopathies, unless it is associated with removal of sickle erythrocytes.¹⁷ In fact, transfusion only increases blood viscosity and further compromises peripheral blood flow. Exchange transfusions are indicated in the acute setting such as stroke, severe acute chest syndrome, or acute priapism.^{8,9,14} For HbSS patients with higher hematocrits (20% or greater in children, 25% or higher in adults), an exchange transfusion technique may be safer than a simple blood transfusion.^{9,14}

The general considerations and indications for blood transfusions in HbSS disease are listed in Table 11-6. Chronic blood transfusions have been shown to reduce the risk of stroke.^{1,24} When discontinuing chronic transfusions becomes necessary (for iron overload concerns, presence of multiple

TABLE 11-5 Effects of Approaches to Specific Therapy

Therapeutic Approach	Drugs	Effect
Inhibition of HbS polymerization	Urea	Noncovalent hemoglobin modification
	Ethanol	
	Peptides	
	Cyanate	Covalent hemoglobin modification
	Pyridoxal	
	Glyceraldehyde	Erythrocyte modification (increase in red cell volume, decrease in 2,3-BPG)
	DDAVP (1-deamino-8-D-arginine vasopressin)	
	Cetiedil	
	Hyponatremia	
	Hydroxyurea	Genetic modification (increase in gamma gene globin expression, bone marrow transplantation)
	5-Azacytidine	
Decreased erythrocyte microvascular entrapment	Nifedipine	Vasodilator

alloantibodies, or other reasons), patients should be carefully followed, because they have an increased risk for recurrent strokes.^{21,24}

Hematopoietic stem cell transplantation (HSCT) is a curative therapy for sickle cell disease.^{8,16} The limitation to HSCT is the lack of human leukocyte antigen (HLA)-matched donors and the risk of graft-versus-host disease, infections, infertility, and other transplant complications.⁸ The results highlighted both the curative potential of bone marrow transplantation and also the severe limitations, which include constrained matched donor availability, a narrow application to the youngest patients in good clinical condition, and rates of transplant-related morbidity and mortality.^{8,16} Nevertheless, as the experience of transplantation for sickle cell disease has expanded, there has been a transition from using this modality as an experimental intervention reserved for those most severely affected to one in which younger children with early signs of sickle-related morbidity are targeted for treatment.^{8,16} Umbilical cord blood units (CBUs) are now considered an acceptable source for hematopoietic stem cell transplantation (HSCT) when HLA-identical donors are unavailable. CBUs have been used successfully in HLA-matched sibling HSCT for children with sickle cell disease.³³

BOX 11-3 Treatment of Sickle Cell Anemia: Goals of Therapeutic Approaches

- Increase the production of fetal hemoglobin in the adult.
- Decrease microvascular entrapment of sickled cells.
- Modify the oxygen affinity or solubility of sickle hemoglobin.
- Change the volume of the sickle erythrocyte.
- Alter expression of the abnormal, sickle gene.
- Prevent endothelial damage.
- Counter oxidant-induced injury.

TABLE 11-6 General Considerations and Indications for Blood Transfusion in Patients With Sickle Cell Anemia

Consideration	Indications
To improve the oxygen-carrying transport in red cells by simple transfusions	<ul style="list-style-type: none"> Severely anemic as reflected by dyspnea, postural hypotension, high-output cardiac failure, angina, or cerebral dysfunction Sudden fall in hemoglobin or hematocrit levels during acute splenic or hepatic sequestration crisis Hemoglobin level <5.0 g/dL or hematocrit of 15% in patients who exhibit fatigue and dyspnea along with erythroid hypoplasia or aplasia
To improve microvascular perfusion by decreasing the number of red cells containing HbS by partial exchange transfusion	<ul style="list-style-type: none"> Life-threatening events, such as cerebrovascular accidents including stroke and transient ischemic attacks (TIAs) Arterial hypoxia syndrome (fat embolization) Acute progressive lung disease Unresponsive acute priapism Eye surgery when performed under local anesthesia and in the nonanemic patient

Source: Charache S, et al (Eds): Management and Therapy of Sickle Cell Disease. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health. NIH Pub 91:2117, August 1991.

It is now possible to efficiently diagnose the presence or absence of sickle cell disease in embryos obtained with in-vitro fertilization, before implantation.²⁷ This powerful technique may help carrier couples by allowing them to select a healthy child without facing the decision of whether or not to abort an affected fetus.

Gene therapy for HbSS disease is also currently being investigated. The goal of gene therapy is to replace the β^s -globin gene with a wild-type β -globin gene for the affected patient to produce healthy cells rather than sickle cells. The success of gene therapy depends on the ability of researchers to isolate, enrich, and insert genes into hematopoietic pluripotent stem cells and generate safe, stable, erythroid-specific replacement gene expression at a level that is sufficient to have a clinical effect.^{34,35,36} Autologous transplantation of genetically corrected hematopoietic stem cells (HSCs) in patients with SCD has been referred to as a gold-standard method, with high-efficacy and low-transplant risks.³⁶ Due to a variety of unique beneficial properties such as lifelong self-renewal ability and multilineage differentiation, the patient's HSCs are the perfect target.³⁶ Gene therapy with HSCs is one of the most attractive treatment options utilizing gene addition or genome editing technologies that can be applied either in vivo or ex vivo within a patient, depending on the treatment.³⁶

ADVANCED CONTENT

Research is being performed to determine the activity of the CRISPR/Cas9 RNA-guided endonuclease as a tool in developing effective and safe homologous recombination-mediated genome editing to cure beta-hemoglobinopathies.^{37,38} CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a type of technology being used to edit genomes. The CRISPR technology allows the ability to alter a DNA sequence and modify the gene function caused by a mutation in the beta-globin gene.³⁸ In 2019, the first sickle cell anemia patient in the United States was treated with CRISPR gene-editing and still remains symptom-free

from sickle cell disease.^{38,39} Approximately 45 patients with sickle cell disease or β thalassemia have been treated with the CRISPR gene-editing technology.³⁹ As a result, CRISPR gene-editing represents another cure for SCD.³⁹

CRITICAL THINKING QUESTION

11-2 Why do individuals who are heterozygous for Hgb S have fewer symptoms than those who are homozygous for Hgb S?

Hemoglobin C Disease and Trait

Hemoglobin C (HbC) disease is found almost exclusively in the black population. HbC differs from normal HbA by the single amino acid substitution of lysine for glutamic acid at the sixth position from the NH_2 terminal end of the β chain (Fig. 11-12).⁴⁰ This represents the same substitution point as in HbS but with a positively charged amino acid. The structural formula for HbC, the presence of which is often referred to as HbC disease, is $\alpha_2\beta_2^{6\text{Glu} \rightarrow \text{Lys}}$. HbC is seen with great frequency in West Africa, particularly northern Ghana, where the incidence is 17% to 28%.⁴⁰ In the United States, only 0.02% of blacks have HbC disease and 2% to 3% are heterozygotes.⁴⁰ The clinical manifestations are mild chronic hemolytic anemia with associated splenomegaly and abdominal discomfort. The red cell morphology is typically normocytic, and normochromic or hyperchromic, with numerous target cells (50% to 90%) and occasionally microspherocytes, fragmented cells, and folded cells⁴⁰ (Fig. 11-13). HbC crystals (Fig. 11-14) or “bar of gold” crystals occur more often in the red cells of individuals who have undergone splenectomy than in those whose spleen is intact.⁴¹ Figure 11-15 is a scanning electron micrograph (SEM) of hemoglobin C crystals. These crystals can be demonstrated in wet preparations by washing the red cells and then suspending them in a sodium citrate solution.⁴²

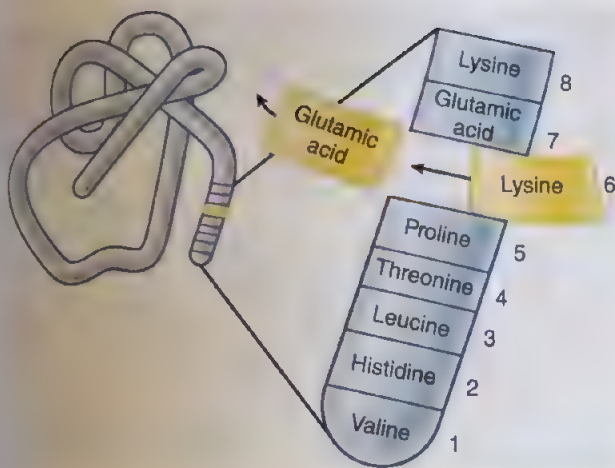


FIGURE 11-12 Amino acid substitution in hemoglobin C.

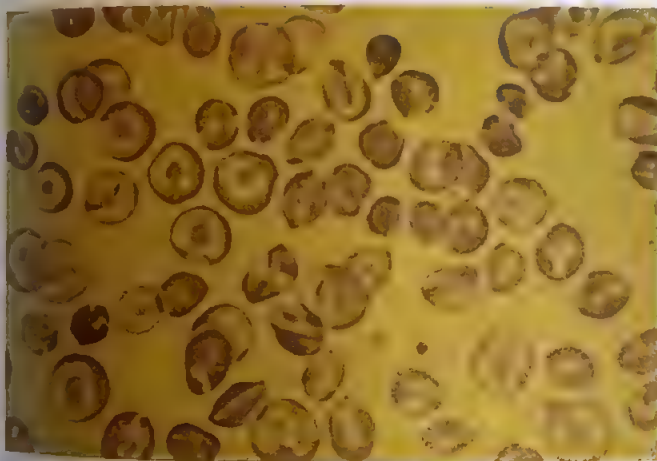


FIGURE 11-13 Hemoglobin C disease (presplenectomy). Note the numerous target and folded cells.

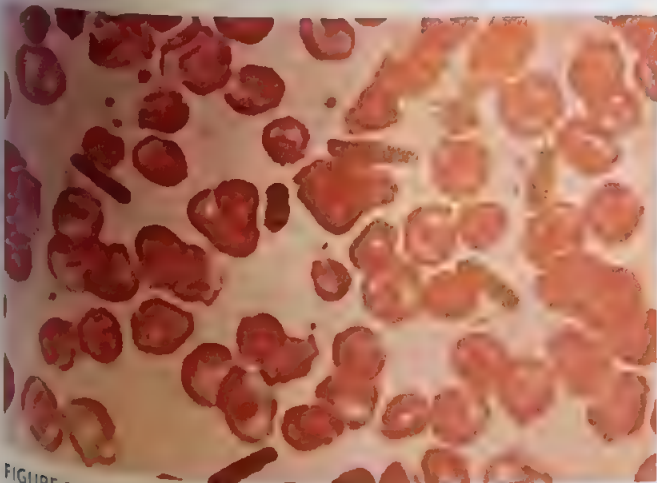


FIGURE 11-14 Hemoglobin C disease (peripheral blood). Note the particular crystals: "bar of gold" and numerous target cells (postsplenectomy). (From Bell A. Hematology. In: Listen, Look and Learn. Bethesda, MD: Health Education Resources, Inc. with permission.)

reticulocyte count is slightly increased. Hemoglobin bands, at alkaline pH, reveal approximately 95% HbC plus A₂, less than 7% HbF, and no HbA. Hemoglobins E, O_{Ara}, C, and A₂ all migrate to the same position at alkaline pH; HbC can be separated from these other hemoglobins at acid pH⁴⁰ (see Fig. 11-10).

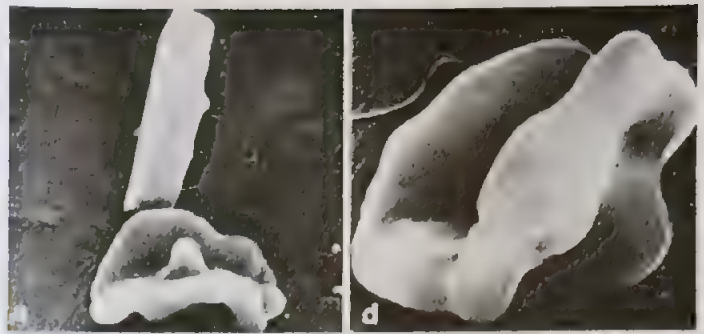


FIGURE 11-15 SEM of hemoglobin C crystals. (From Bell A. Hematology. In: Listen, Look and Learn. Bethesda, MD: Health Education Resources, Inc. with permission.)

HbC trait, $\alpha_2\beta_1\beta_1^{6\text{Glu-Lys}}$, is present in 2% to 3% of American blacks, and these individuals are clinically asymptomatic.⁴⁰ The only significant finding on the peripheral blood smear is targeting. At alkaline pH, there is approximately 60% HbA and 40% HbC plus A₂.⁴⁰

Hemoglobin D Disease and Trait

Hemoglobin D (HbD) disease has several variants. The most common variant in American blacks is HbD_{Punjab}, which is synonymous with HbD_{Los Angeles}. It was also known as HbD-North Carolina, HbD-Portugal, HbD-Chicago, and Hb Oak Ride.⁴² HbD-Punjab is prevalent in Pakistan, Northwest India, China, and Middle East countries where its frequency is 0.2% to 3.0%.⁴² In the United States, the frequency is less than 0.02%.⁴² Both the homozygous ($\alpha_2\beta_2^{121\text{Glu-Gln}}$) and the heterozygous ($\alpha_2\beta_1\beta_1^{121\text{Glu-Gln}}$) states (where glycine is substituted for glutamic acid) are asymptomatic. The peripheral blood smear is unremarkable, except for a few target cells. HbD migrates electrophoretically to the same position as HbS and HbG at alkaline pH but migrates with HbA at acid pH. HbD is a nonsickling soluble hemoglobin.⁴²

Hemoglobin E Disease and Trait

Hemoglobin E (HbE) disease occurs with greatest frequency in Burma, Thailand, Cambodia, Laos, Malaysia, and Indonesia with an incidence of 15%.⁴³ In the United States, it is a rare disease.⁴³ The homozygous state ($\alpha_2\beta_2^{26\text{Glu-Lys}}$) presents with little or no anemia, target cells, and microcytic, hypochromic red cell indices.⁴³ Alkaline electrophoresis reveals approximately 95% to 97% HbE plus A₂, and the remainder of the hemoglobin is HbF. HbE migrates with HbC and HbO_{Ara} at alkaline pH but migrates with HbA at acid pH. HbE trait ($\alpha_2\beta_1\beta_1^{26\text{Glu-Lys}}$) is asymptomatic clinically. Microcytosis, target cells, and approximately 70% HbA and 30% HbE plus A₂ are noted on routine electrophoresis.⁴³ HbE is slightly unstable, and there is an associated thalassemic component with this hemoglobin variant. This is responsible for the microcytosis and the lower-than-expected quantified value of HbE in HbAE.⁴⁴

It has been postulated that HbE may protect against malaria, because areas such as Thailand that are highly endemic for malaria also have a high incidence of the HbE gene. Some authors attribute this effect to the fact that the parasite *Plasmodium falciparum* multiplies more slowly in HbE red cells than in the HbAE or HbAA red cells.⁴⁵

Hemoglobin O_{Arab} Disease and Trait

Hemoglobin O_{Arab} (HbO_{Arab}) disease is a rare inherited hemoglobin mutation in the β -globin gene that occurs in individuals of Balkan or North African black descent.^{45,46} Homozygous O_{Arab} ($\alpha_2\beta_2^{121\text{Glu-Lys}}$) disease exhibits a mild hemolytic anemia with slight splenomegaly and target cells on the peripheral blood smear.⁴⁶ This hemoglobin migrates electrophoretically with HbC, HbE, and HbA₂ at alkaline pH but separates at acid pH, migrating in the HbA position (see Fig. 11-10). In the heterozygous state of HbO_{Arab} ($\alpha_2\beta_1\beta_1^{121\text{Glu-Lys}}$), the patient is asymptomatic.⁴⁶

Hemoglobin S With Other Abnormal Hemoglobins

As mentioned previously, sickle cell disease is a generic term for a group of genetic disorders that includes sickle cell anemia and hemoglobinopathies in which HbS is found in association with another abnormal hemoglobin, and the sickle β -thalassemia syndromes.⁴⁷ This section focuses on defining the disorders that have HbS and another abnormal hemoglobin. Sickle β -thalassemia is briefly mentioned at the end of this section. The common and uncommon forms of sickle cell disease are listed in Table 11-7.

Hemoglobin SC Disease

Hemoglobin SC (HbSC) disease ($\alpha_2\beta_1^{6\text{Val}}\beta_1^{6\text{Glu-Lys}}$) occurs when the gene for HbS is inherited from one parent and that for HbC from the other.⁴⁸ About 0.12% of black Americans have SC disease.⁴⁹ Patients with HbSC disease are generally less anemic and experience a milder course than those with HbSS disease. However, because of increased blood viscosity, this condition has a greater incidence of cerebral vasculopathy, retinal hemorrhage, renal papillary necrosis, and necrosis of the femoral head.^{48,50}

Peripheral blood smear findings include target cells, folded red cells, and occasionally glove-shaped intracellular crystals (Figs. 11-16 and 11-17).⁴⁸ The solubility test results are positive owing to the presence of HbS. Hemoglobin electrophoresis at alkaline pH separates HbS and HbC in approximately equal amounts (Fig. 11-18). HbF is usually less than 2% compared with average HbF levels of about 6% in sickle cell anemia.⁵¹ Electrophoresis at acid pH confirms the S and C hemoglobins (see Fig. 11-10). Table 11-8 compares the incidence of the most common hemoglobinopathies found in American blacks.

TABLE 11-7 Common and Uncommon Forms of Sickle Cell Disease

Common	Uncommon
(HbSS) sickle cell anemia	(HbSD) hemoglobin SD disease
(HbSC) sickle-HbC disease	(HbSO _{Arab}) hemoglobin SO _{Arab}
(HbS β^0) sickle β^0 thalassemia	(HbSE) hemoglobin SE disease
(HbS β^0) sickle β^0 thalassemia	(HbS-Lepore) hemoglobin S Lepore

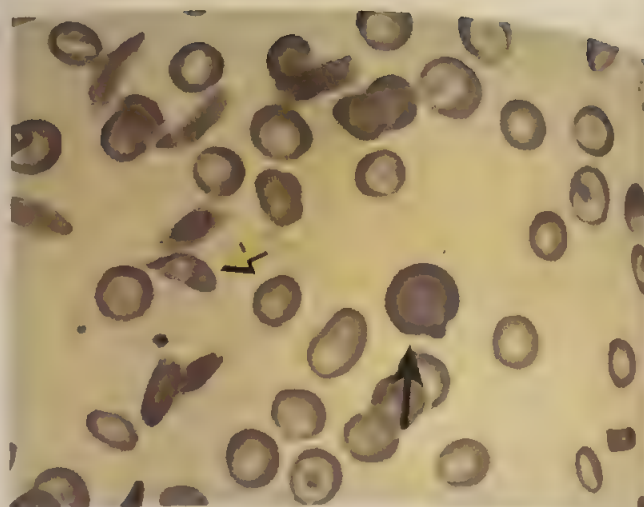


FIGURE 11-16 Hemoglobin SC disease (peripheral blood). Note the formation of the SC crystal (yellow arrow) and the polychromasia (black arrow).

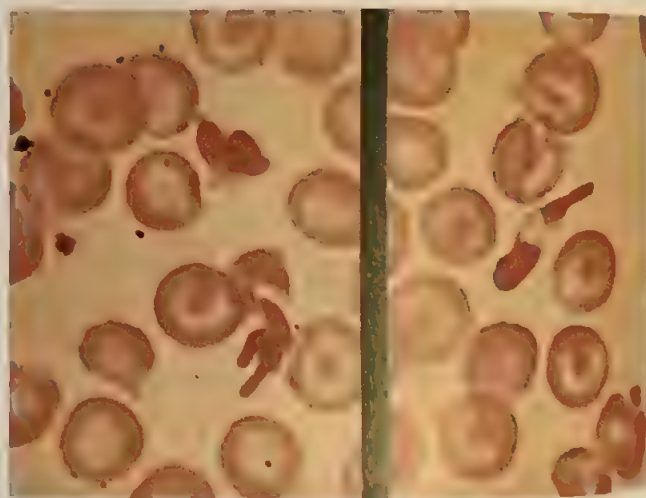


FIGURE 11-17 Hemoglobin SC disease (peripheral blood). Note the type of "Washington Monument" crystals and target cells. (From Bell A. Hematology. In: Listen, Look and Learn. Bethesda, MD: Health Education Resources, Inc. with permission.)

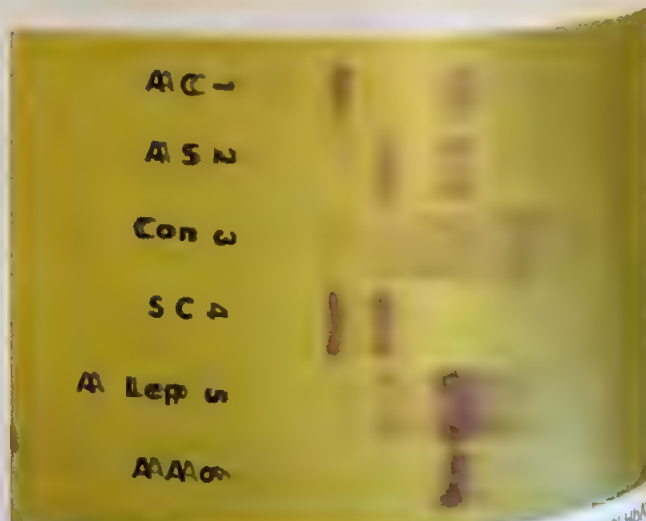


FIGURE 11-18 Hemoglobin electrophoretic patterns: (1) HbA_{1c}, (2) HbA₂, (3) commercial control, (4) HbSC, (5) HbA-Lepore (see Chapter 12), and (6) HbAA normal control.

TABLE 11-8 Incidence of Common Hemoglobinopathies in American Blacks

Condition	Genotype	Incidence (All Ages)
Hemoglobin C disease	$\alpha_2\beta^c\beta^c$	0.02% (1 in 4,500)
Hemoglobin C trait	$\alpha_2\beta^A\beta^c$	3.0% (1 in 33)
Sickle cell anemia	$\alpha_2\beta^s\beta^s$	0.26% (1 in 375)
Sickle cell trait	$\alpha_2\beta^A\beta^s$	8.0% (1 in 13)
Sickle C disease	$\alpha_2\beta^s\beta^c$	0.12% (1 in 835)
Sickle β thalassemia	$\alpha_2\beta^s\beta^0$	0.06% (1 in 1,667)

Hemoglobin SD Disease

The combination of HbS and HbD disease, although rare, presents an interesting diagnostic problem.⁵² Because these hemoglobins migrate together at alkaline pH, the electrophoretic pattern is similar to that of HbSS disease.⁵² Solubility tests are positive.

The clinical severity of HbSD disease, however, falls between that of sickle cell anemia and that of sickle cell trait.⁵² Acid electrophoresis separates these two hemoglobins. HbS has its own migration point, whereas HbD migrates with HbA in the same position (see Fig. 11–10).⁵²

Hemoglobin SO_{Arab} and S-Oman Disease

The combination of HbS and HbO_{Arab} disease can have a clinical presentation that is similar in severity to that of HbSS disease.⁵³ The anemia is severe, with typical sickled cells seen on the peripheral blood smear.⁵³ This condition might initially be confused with HbSC on routine electrophoresis; however, differentiation can be made with acid electrophoresis (see Fig. 11–10).

Hemoglobin S-Oman (HbS-Oman) is a unique hemoglobin variant that carries both the classic sickle mutation in β^6 and one additional mutation in $\beta^{121}\text{Glu-Lys}$, which is the same substitution observed in HbO_{Arab}.⁵⁴ Heterozygotes of HbA/S-Oman genotype with HbS-Oman levels of 20% or less present with some clinical complications of sickle cell disease.⁵⁴

Hemoglobin S/ β -Thalassemia Combination

The severity of HbS combined with β thalassemia depends on the degree of suppression of β -globin chain synthesis.⁴⁷ Hemoglobin S/ β^0 thalassemia is a severe condition that clinically resembles sickle cell anemia; on the other hand, HbS/ β^+ thalassemia generally has a milder clinical presentation.⁴⁷ The reader is referred to Chapter 12 for a detailed discussion of HbS/ β thalassemia and other hemoglobin variants that occur in combination with thalassemia.

Laboratory Diagnosis of HbS With Other Abnormal Hemoglobins

When HbS is found in association with another abnormal hemoglobin, the diagnosis in many instances can be made from hemoglobin electrophoresis alone. However, it may be difficult to distinguish between HbSS disease and some of the sickle β -thalassemia syndromes such as HbS β^0 thalassemia,

HbS β^+ thalassemia, HbS $\delta\beta$ thalassemia, and HbS in association with hereditary persistence of fetal hemoglobin syndrome (HbS HPFH). In these cases, the electrophoresis demonstrates only HbS, HbF, and HbA₂. It is important to properly diagnose these disorders because the clinical manifestations and subsequent treatment are different. For example, HbS β^0 thalassemia is similar in severity to HbSS disease. Patients with HbS $\delta\beta$ thalassemia have few symptoms; HbS/ β^+ thalassemia is a milder form, and HbS HPFH is usually asymptomatic with no anemia. Measurement of HbF and HbA₂ may be helpful in distinguishing these conditions because patients with HbSS, HbS β^0 thalassemia, HbS $\delta\beta$ thalassemia, and HbS HPFH all have similar electrophoretic patterns. In HbS- β^0 thalassemia, HbA₂ levels are greater than 3.5%, whereas they are low in patients with HbS $\delta\beta$ thalassemia and HbS HPFH.^{6,55} Generally, HbF levels are higher in all the HbS β thalassemias in comparison with HbSS. Assessment of HbF in the parents may be indicated when HbS HPFH is suspected. The current gold standard for hemoglobin variant identification is High Performance Liquid Chromatography (HPLC); however, a state-of-the-art laboratory infrastructure and highly trained personnel are necessary. Currently, hemoglobin variant identification using point-of-care microchip electrophoresis has emerged.⁵⁵ The paper-based microchip electrophoresis technology helps with diagnosis of hemoglobin disorders in resource-limited settings.^{56,57} Paper-based microchip electrophoresis can provide low-cost, rapid, reproducible, and accurate point-of-care tests for hemoglobin analysis. This technology is ideal for low- and middle-income countries and for newborn screening.^{55,56,57,58}

The clinical and hematologic findings in the common variants of sickle cell disease are summarized in Table 11–9.

CRITICAL THINKING QUESTION

11-3 Why is electrophoresis the most effective laboratory analysis for the diagnosis of HbS with other abnormal hemoglobins?

ADVANCED CONTENT

Hemoglobin Variants with Altered Oxygen Affinity

Hemoglobin variants with altered oxygen affinity are caused by mutations of the globin genes.⁵⁹

High-affinity hemoglobins, which are inherited as an autosomal dominant disorder, are seen in the heterozygous state. These hemoglobins bind oxygen more readily and release it less easily to the tissues. The result is tissue hypoxia, which stimulates increased EPO production. This, in turn, causes a compensatory increase in red cell mass, with increases in red cell count, hemoglobin, and hematocrit, producing erythrocytosis. Other hematologic parameters are normal. There is a shift to the left in the oxygen dissociation curve, and a diagnosis

TABLE 11-9 Clinical and Hematologic Findings in the Common Variants of Sickle Cell Disease After the Age of 5 Years

Disease Group	Clinical Severity	Hemoglobin Electrophoresis				Hematologic Values*			
		S (%)	F (%)	A ₁	A (%)	Hb (g/dL)	Retic (%)	MCV (fL)	RBC Morphology
SS	Usually marked	>90	<10	<3.5	0	6–10	5–20	>80	Sickle cells nRBCs Normochromic, normocytic Anisocytosis Poikilocytosis Target cells Howell–Jolly bodies
S ⁰ Thal	Marked to moderate	>80	<20	>3.5	0	6–10	5–20	<80	Sickle cells nRBCs Hypochromic Microcytosis Anisocytosis Poikilocytosis Target cells
S ⁺ Thal	Mild to moderate	>60	<20	<3.5	20 (A)	9–12	5–10	<75	No sickle cells Hypochromic Microcytosis Anisocytosis Poikilocytosis Target cells
SC	Mild to moderate	50	<5	50 (C)	0	10–15	5–10	75–95	Occasional SC crystals Anisocytosis Poikilocytosis Target cells
S HPFH	Asymptomatic	>70	>30	<2.5	0	12–14	1–2	<75	No sickle cells Anisocytosis Poikilocytosis Rare target cells

*Hematologic values are approximate. There is a tremendous variability between disease groups and between individual patients of the same group, particularly with regard to clinical severity.

SS = sickle cell anemia; S⁰Thal = sickle beta zero thalassemia; S⁺Thal = sickle beta plus thalassemia; S HPFH = HbS in association with the hereditary persistence of fetal hemoglobin syndrome; SC = hemoglobin SC disease; nRBCs = nucleated red blood cells.

Source: Charache S, et al (Eds) Management and Therapy of Sickle Cell Disease. US Department of Health and Human Services, Public Health Service, National Institutes of Health. NIH Pub 91-2117, August 1991.

is established by measuring P_{50} levels (Fig. 11-19). High oxygen affinity variants have a low P_{50} .⁴⁹ Individuals with these hemoglobin variants are asymptomatic.⁴⁹ For a review of P_{50} , refer to Chapter 2.

Hemoglobins with decreased oxygen affinity release oxygen quite readily to the tissues. There is a shift to the right in the oxygen-dissociation curve and the low oxygen affinity variants have a high P_{50} (see Fig. 11-19).⁴⁹ As

more oxygen is released per gram of hemoglobin, P_{50} concentrations fall. This can result in decreased hemoglobin concentration with the development of a mild anemia. There may also be mild cyanosis associated with a decreased oxygen saturation level. Hemoglobins with increased or decreased oxygen affinities are listed in Table 11-10. In cases of unexplained erythrocytosis or cyanosis, oxygen affinity studies may be helpful.

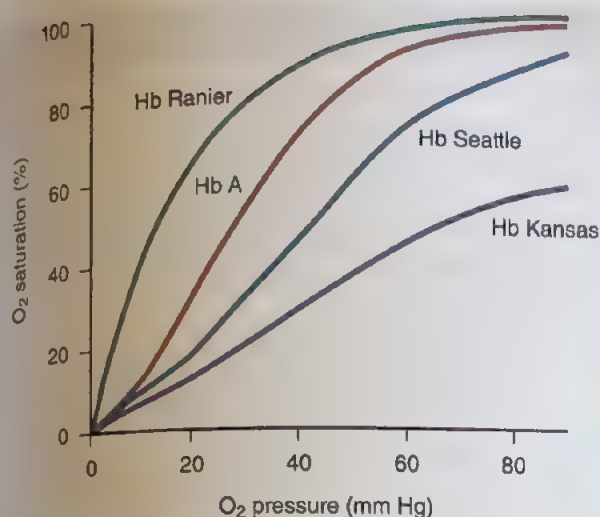


FIGURE 11-19 Oxygen equilibrium curve of whole blood from subjects with Hb Rainier, Hb Seattle, Hb Kansas, and normal control (HbA).

TABLE 11-10 Hemoglobins Associated With Altered Oxygen Affinity

Increased O ₂ Affinity and Polycythemia		Decreased O ₂ Affinity—May Have Mild Anemia or Cyanosis	
Hb _{Chesapeake}	$\alpha_2\beta_2^{92Leu}$	Hb _{Kansas}	$\alpha_2\beta_2^{102Thr}$
Hb _{Cape Town}	$\alpha_2\beta_2^{92Gln}$	Hb _{Titusville}	$\alpha_2\beta_2^{94Asn}$
Hb _{Maine}	$\alpha_2\beta_2^{97Gln}$	Hb _{Providence}	$\alpha_2\beta_2^{82Asn, Asp}$
Hb _{Leucoma}	$\alpha_2\beta_2^{99His}$	Hb _{Aggenogi}	$\alpha_2\beta_2^{90Lys}$
Hb _{Kempsey}	$\alpha_2\beta_2^{99Asn}$	Hb _{Beth Israel}	$\alpha_2\beta_2^{102Ser}$
Hb _{eps-Tyroland}	$\alpha_2\beta_2^{99Tyr}$	Hb _{Yoshizuka}	$\alpha_2\beta_2^{108Asp}$
Hb _{Marshall}	$\alpha_2\beta_2^{146Asp}$	Hb _{Seattle}	$\alpha_2\beta_2^{70Asp}$
Hb _{Rainier}	$\alpha_2\beta_2^{145Cy}$		
Hb _{Bethesda}	$\alpha_2\beta_2^{145His}$		

Unstable Hemoglobins

More than 150 unstable hemoglobins (Fig. 11-20) associated with hemolytic anemias have been described.⁶⁰ Unstable hemoglobins are hemoglobin variants in which amino acid substitutions or deletions have weakened the binding forces that maintain the structure of the molecule.⁶⁰ The instability may cause hemoglobin to denature and precipitate in the red cells as Heinz bodies. Most unstable hemoglobin variants are rare inherited autosomal dominant disorders.⁶⁰ However, absence of a positive family history is not always helpful, as new mutations are common. Many mutations producing unstable hemoglobinopathies are single amino acid substitutions in either the α -, β -, γ -, or δ -globin chains that affect a few key areas of the hemoglobin structure. By far, the majority of these substitutions are in the β -globin chain, followed by an α -chain substitution and only a few in γ or δ chains.⁶⁰ The unstable hemoglobin results from amino acid substitutions within the heme pocket of the alpha or beta polypeptide chains period.



FIGURE 11-20 Unstable hemoglobin: Hb Zurich (peripheral blood). (From Bell A. Hematology. In: Listen, Look and Learn. Bethesda, MD: Health Education Resources, Inc. with permission.)

The mode of inheritance is typically autosomal dominant.⁶⁰ Many of the unstable hemoglobins have high oxygen affinity and, therefore, may not cause anemia, making diagnosis in this group of patients difficult. When anemia is present, the degree of hemolysis associated with an unstable hemoglobin varies considerably.⁶⁰ Some patients experience severe chronic hemolysis with jaundice and splenomegaly. However, most patients have a mild compensated condition and seek medical attention only after exacerbation of the hemolysis caused by infection and increased temperature or exposure to oxidative drugs.⁶⁰ Reticulocytosis is variable. Hypochromia may be apparent on the peripheral blood smear, and the mean red cell hemoglobin (MCH) content can be low in some cases because the unstable hemoglobin may be denatured and “pitted” out of the cell by the mononuclear phagocytic cells of the spleen.

Hemoglobin electrophoresis IEF or HPLC is usually not a very helpful laboratory method to detect unstable hemoglobins; however, subtle indications of an abnormality may be observed.⁶⁰ These include an increased level of HbA₂, a common finding in unstable β -chain hemoglobins, and increased HbF (Table 11-11).⁶⁰

Most hospitals still perform the isopropanol stability or heat stability test for detection of unstable hemoglobins.⁶⁰ The only test that may detect some of the rare unstable hemoglobins is globin gene sequencing. In affected neonates, sequencing the globin genes, including the gamma globin gene, is often needed for a definitive diagnosis.⁶¹

Methemoglobinemia

Methemoglobinemia is a clinical condition with methemoglobin levels greater than 1% of the total hemoglobin.⁶² It is a rare disorder in which the heme contains the oxidized ferric form of iron (Fe^{3+}) rather than the ferrous form (Fe^{2+}) forming methemoglobin.^{63,64} In this state, the molecule is unable to bind oxygen, which results in cyanosis. The blood is chocolate-brown in color. In general, there are three causes of methemoglobinemia:^{63,64, 65}

1. Hemoglobin M variants (autosomal dominant variants in the globin genes)

TABLE 11-11 Unstable Hemoglobins*

α -Chain Abnormalities		β -Chain Abnormalities	
Hb _{Torina}	$\alpha_2^{43\text{Val}}\beta_2$	Hb _{Leiden}	$\alpha_2\beta_2^{60\text{7}}$ (Glu deleted)
Hb _{L-Ferrara}	$\alpha_2^{47\text{Gly}}\beta_2$	Hb _{Sogn}	$\alpha_2\beta_2^{14\text{A}}$
Hb _{Hasharon}	$\alpha_2^{47\text{His}}\beta_2$	Hb _{Freiburg}	$\alpha_2\beta_2^{23}$ (Val deleted)
Hb _{Ann Arbor}	$\alpha_2^{80\text{Arg}}\beta_2$	Hb _{Riverdale Bronx}	$\alpha_2\beta_2^{24\text{Arg}}$
Hb _{Etobicoke}	$\alpha_2^{84\text{Arg}}\beta_2$	Hb _{Genova}	$\alpha_2\beta_2^{28\text{Pro}}$
Hb _{Dakar}	$\alpha_2^{112\text{Gln}}\beta_2$	Hb _{Tacoma}	$\alpha_2\beta_2^{30\text{Ser}}$
Hb _{Bibba}	$\alpha_2^{136\text{Pro}}\beta_2$	Hb _{Philly}	$\alpha_2\beta_2^{35\text{Phe}}$
		Hb _{Louisville}	$\alpha_2\beta_2^{42\text{Leu}}$
		Hb _{Hammersmith}	$\alpha_2\beta_2^{42\text{Ser}}$
		Hb _{Zurich}	$\alpha_2\beta_2^{63\text{Arg}}$
		Hb _{Toulouse}	$\alpha_2\beta_2^{66\text{Glu}}$
		Hb _{Bristol}	$\alpha_2\beta_2^{67\text{Asp}}$
		Hb _{Sydney}	$\alpha_2\beta_2^{67\text{Ala}}$
		Hb _{Shepherd's Bush}	$\alpha_2\beta_2^{74\text{Asp}}$
		Hb _{Seattle}	$\alpha_2\beta_2^{70\text{Asp}}$
		Hb _{Boras}	$\alpha_2\beta_2^{88\text{Arg}}$
		Hb _{Santa Ana}	$\alpha_2\beta_2^{88\text{Pro}}$
		Hb _{Gun Hill}	$\alpha_2\beta_2^{91-95}$ (5 a.a. deleted)
		Hb _{Sabine}	$\alpha_2\beta_2^{91\text{Pro}}$
		Hb _{Kolin}	$\alpha_2\beta_2^{98\text{Met}}$
		Hb _{Kansas}	$\alpha_2\beta_2^{102\text{Thr}}$
		Hb _{Wein}	$\alpha_2\beta_2^{130\text{Asp}}$
		Hb _{Olmsted}	$\alpha_2\beta_2^{141\text{Arg}}$

*Hemoglobins that may precipitate as Heinz bodies after splenectomy: congenital Heinz body hemolytic anemia. a.a. = amino acids.

2. NADH-methemoglobin reductase deficiency (autosomal recessive inheritance; see Chapter 10)
3. Toxic substance⁶⁶ (acquired; see Chapter 10)

There are five variants of hemoglobin M (Table 11-12), which result from a single amino acid substitution in the α - or β - and γ -globin chains that stabilizes iron in the ferric form.^{63,64} A substitution of a tyrosine amino acid for either the proximal (F8) or the distal (E7) histidine amino acid in the α , β , or γ chains is involved for most M hemoglobins.⁶⁶ These substitutions cause heme iron to auto-oxidize, which results in methemoglobinemia. If the substitution occurs in the α chain, cyanosis is present at birth. Cyanosis does not occur with a β -chain substitution until approximately 6 months of age.⁶⁴ This correlates with the switch from γ to β chains. The presumptive diagnosis of HbM is made from the absorption spectra of hemolysates and hemoglobin electrophoresis on agar gel at pH 7.1.^{61,64} Patients have obvious cyanosis but otherwise are generally asymptomatic. No specific treatment is indicated or possible.

For cases primarily caused secondary to drug exposure, methylene blue is used to reverse the bound ferric iron (Fe³⁺) of methemoglobin to ferrous iron (Fe²⁺).^{62,63} In addition to methylene blue, ascorbic acid, riboflavin, hyperbaric oxygen therapy, and even RBC transfusion (for refractory or severe cases) are used as treatments for methemoglobinemia.^{62,63}

TABLE 11-12 Hemoglobins Associated With Methemoglobinemia and Cyanosis

Variant	Hemoglobin
HbM _{Boston}	$\alpha_2^{80\text{Y}}\beta_2$
HbM _{Wate}	$\alpha_2^{81\text{Y}}\beta_2$
HbM _{Saskatoon}	$\alpha_2\beta_2^{61\text{Y}}$
HbM _{Milwaukee}	$\alpha_2\beta_2^{62\text{Y}}$
HbM _{Hyde Park}	$\alpha_2\beta_2^{92\text{Y}}$

SUMMARY CHART

- More than 90% of the hemoglobin variants are single amino acid substitutions in the alpha (α)-, beta (β)-, delta (δ)-, or gamma (γ)-globin chains as a result of a single-point mutation.
- Hemoglobin S (HbS) is produced when valine substitutes for glutamic acid in the sixth position of the β chain.
- Hemoglobin C (HbC) is produced when lysine replaces glutamic acid at position six of the β chain.
- Sickle cell anemia (HbSS disease) is the most common type of sickle cell disease and represents the homozygous form in which the individual inherits a double dose of the abnormal gene that codes for HbS.
- The characteristic morphology in HbSS disease is the sickled cell, which increases the viscosity of the blood, leading to hypoxia, painful crises, and infarction of the spleen, kidney, and bone marrow.
- The three types of crises associated with sickle cell disease are aplastic, hemolytic, and vaso-occlusive (painful).
- In sickle cell disease, HbS constitutes 80% or more of the hemoglobin content in addition to HbF plus A₂.
- Sickle cell trait represents the heterozygous form of sickle cell disease in which individuals inherit both a normal β -globin gene and a sickle globin gene.
- Individuals with sickle cell trait produce both HbA and HbS in a ratio of 60:40.
- In sickle cell disease (HbSS) the red blood cell (RBC) indices are normochromic and normocytic, with hemoglobins ranging from 6 to 8 g/dL, target cells, sickled cells, nucleated red cells, and polychromasia.
- The definitive test for HbS is hemoglobin electrophoresis on cellulose acetate and citrate agar.
- HbC disease presents with a normocytic, and normochromic or hyperchromic anemia, and numerous target cells, microspherocytes, schistocytes, folded cells, and "bar of gold" crystals.
- Electrophoresis in HbC disease shows 95% HbC plus A₂, and less than 7% HbF.
- Hemoglobin SC (HbSC) disease occurs when an HbS gene is inherited from one parent and an HbC gene is inherited from the other; morphology includes target cells, folded red cells, and glove-shaped intracellular crystals.
- Methemoglobinemia occurs when levels exceed 1% of total hemoglobin and may be the result of hemoglobin M (HbM) variants, NADH-diaphorase deficiency, or toxic substances.

CASE STUDY 11-1

A 13-year-old girl, in the black community was admitted to the hospital appearing acutely ill with fever and abdominal pain. On physical examination, an enlarged spleen was evident. Laboratory test results were as follows:

Hgb	5.0 g/dL
Hct	15%
RBC count	$1.4 \times 10^{12}/L$
WBC count	$2.2 \times 10^9/L$
Reticulocyte count	1%
Differential	
Segmented neutrophils	62%
Bands	12%
Lymphocytes	19%
Monocytes	4%
Eosinophils	2%
Basophils	1%
RBC indices	Normal
Platelet count	$400 \times 10^9/L$
Peripheral blood smear	(see Fig. 11-16)

position. The hemoglobins were quantified as 55% HbS and 45% HbC plus A₂. Hemoglobins S and C were confirmed by electrophoresis at acid pH.

QUESTIONS

1. Does the CBC reveal anemia?
2. Describe the morphological features of the red blood cells that were likely seen on the peripheral blood smear of this patient.
3. In reviewing the electrophoretic data, what diagnosis is suggested?
4. Comment on crystal formation in this condition.
5. Discuss the clinical presentation of the patient. Is it consistent with HbSC disease?
6. This girl's parents have no hematologic problems; therefore, for her to have HbSC disease, what would be their most likely genotypes?
7. The inheritance of structurally abnormal hemoglobins follows simple mendelian laws. With parents having the trait form of HbS and HbC, what would be the expected genotypes in any of four children?

Hemoglobin electrophoresis, alkaline pH, showed one band in the HbS position and one band in the HbC

Continued

CASE STUDY 11-1—cont'd

ANSWERS

1. Yes, there is a low RBC count with low Hgb and Hct values.
2. Numerous target cells are likely present on the peripheral blood smear along with some cells that appear to have shadows of precipitating intraerythrocytic crystals.
3. The data suggest a diagnosis of HbSC disease with equally strong HbS and HbC bands present.
4. The crystals in HbSC disease appear to be only partially formed, or there may be more than one formation. The crystals in the red cells often are described as having a glove-shaped appearance with several "fingers" protruding.
5. Generally, HbSC disease has a milder presentation than HbSS disease; however, this patient appears to be experiencing a severe episode of SC crisis. This is indicated by her acute illness, abdominal pain, and decrease in hemoglobin and hematocrit without an increase in the reticulocyte count.
6. With no hematologic problems, one parent would be expected to have HbS trait (HbAS), whereas the other would most likely have HbC trait (HbAC).
7. The probability for each birth would be 25% for HbAA, 25% for HbAS, 25% for HbAC, and 25% for HbSC.

REVIEW QUESTIONS

1. Which of the following is true regarding the composition of hemoglobin?
 - a. Hemoglobin is a polysaccharide.
 - b. Hemoglobin contains six polypeptide chains in one molecule.
 - c. The heme groups are attached to the globin portions.
 - d. The heme portion is composed of globin and iron.
2. For proper oxygen transport, iron needs to be in the ____ state.
 - a. Ferric
 - b. Ferrous
 - c. Conjugated
 - d. Bound
3. The most common globin chain abnormalities causing hemoglobinopathies are:
 - a. Alpha chain abnormalities
 - b. Beta chain abnormalities
 - c. Gamma chain abnormalities
 - d. Epsilon chain abnormalities
4. Which statement is true of qualitative hemoglobinopathies?
 - a. They result from decreased production of hemoglobin.
 - b. They result from increased production of hemoglobin.
 - c. They result from abnormal sequencing of amino acids in the globin chain.
 - d. They result from decreased synthesis of globin.
5. What is the amino acid substitution found in sickle cell anemia?
 - a. Substitution of valine for glutamic acid in the sixth position from the NH₂-terminal ⊕ β chain
 - b. Substitution of lysine for glutamic acid in the sixth position from the NH₂-terminal ⊕ β chain
 - c. Substitution of lysine for glutamic acid in the 26th position from the NH₂-terminal ⊕ β chain
 - d. Substitution of valine for glutamic acid in the 121st position from the NH₂-terminal ⊕ β chain
6. What factors contribute to the sickling of RBCs?
 - a. Increase in pH and oxygenation
 - b. Decrease in pH and oxygenation, and dehydration
 - c. Increase in pH and decrease in oxygenation
 - d. Decrease in dehydration and increase in pH and oxygenation
7. Which is true of sickle cell disease?
 - a. Often diagnosed later in life.
 - b. Presents as normocytic, normochromic anemia.
 - c. Hemoglobin levels are often normal.
 - d. Patients have increased risk of tissue injury due to vaso-occlusion.
8. Why is antibiotic therapy an effective treatment for sickle cell disease?
 - a. It rehydrates the patient to combat sickling of red cells.
 - b. It provides pain relief.
 - c. It replaces hemoglobin levels
 - d. It treats underlying infections that often precipitate a crisis.
9. Why is *S. pneumoniae* a dangerous bacteria for sickle cell patients?
 - a. Splenic dysfunction predisposes patients to infection with encapsulated bacteria.
 - b. Anaerobic bacteria can cause infections in sickle cell disease.
 - c. Sickled cells can't illicit a proper immune response.
 - d. Decreased neutrophil counts give patients a lesser immune reaction.
10. Which of the following laboratory results is expected in sickle cell disease?
 - a. Normal hemoglobin levels
 - b. Hemoglobin levels of 10 to 12 mg/dL
 - c. Hemoglobin levels of 6 to 8 mg/dL
 - d. Increased hemoglobin levels

REVIEW QUESTIONS—cont'd

11. What is the amino acid substitution found in HbC disease?
 - a. Substitution of valine for glutamic acid in the sixth position from the NH₂-terminal \oplus β chain
 - b. Substitution of lysine for glutamic acid in the sixth position from the NH₂-terminal \oplus β chain
 - c. Substitution of lysine for glutamic acid in the 26th position from the NH₂-terminal \oplus β chain
 - d. Substitution of valine for glutamic acid in the 121st position from the NH₂-terminal \oplus β chain
12. Which is seen on the peripheral smear of patients with HbC disease?
 - a. Sick cells
 - b. Target cells
 - c. Heinz bodies
 - d. Significantly increased retics
13. Glycine is substituted for _____ in HbD disease?
 - a. Glutamic acid
 - b. Lysine
 - c. Valine
 - d. Porphyrin
14. Which cell is seen in HbE disease?
 - a. Target cells
 - b. Macrocytes
 - c. Sick cells
 - d. Schistocytes
15. Which cells are seen in HbO_{Arab} disease?
 - a. Target cells
 - b. Macrocytes
 - c. Sick cells
 - d. Schistocytes
16. HbS and HbC separate on which lab analysis, helping to diagnose HbSC disease?
 - a. Acid pH electrophoresis
 - b. Acid elution test
 - c. Alkaline pH electrophoresis
 - d. Antiglobulin test
17. Which of the following characterize unstable hemoglobins?
 - a. Hemoglobin variants in which amino acid substitutions or deletions have weakened the binding forces that maintain the structure of the hemoglobin molecule.
 - b. Most unstable hemoglobins are inherited recessive disorders.
 - c. The majority of the unstable hemoglobin single amino acid substitutions are in the delta globin chains.
 - d. The method of choice to detect unstable hemoglobins is hemoglobin electrophoresis.
18. Methemoglobin levels greater than what percent define the clinical condition of methemoglobinemia?
 - a. 10%
 - b. 20%
 - c. 1%
 - d. 5%

See answers at the back of this book.

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Hemolytic Anemias

Intracorpuseular Defects: Thalassemia

Samantha J. Peterson, PhD, MS, MLS(ASCP)^{CM} • Russell Aaron Higgins, MD

CHAPTER OUTLINE

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A Broad Clinical Classification of
Thalassemia Syndrome

Pathophysiology

Thalassemia Syndromes

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Alpha Thalassemia
Other Thalassemia and Thalassemia-
like Conditions

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Routine Hematology Procedures
Flow Cytometry
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LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- | | |
|--|--|
| <p>12-1 Identify the normal human hemoglobins and their composition.</p> <p>12-2 Define thalassemia.</p> <p>12-3 Describe the hemoglobin alteration that leads to thalassemia.</p> <p>12-4 Explain the genetic defects related to thalassemia.</p> <p>12-5 Describe the clinical expression of different gene combinations of α and β thalassemia.</p> <p>12-6 Compare and contrast the α and β thalassemias, including their etiology, pathophysiology, clinical presentation, and laboratory findings.</p> | <p>12-7 Describe the condition of Hereditary Persistence of Fetal Hemoglobin (HPFH).</p> <p>12-8 List the hallmark laboratory findings associated with thalassemia, including CBC values, hemoglobin electrophoresis results, and peripheral blood smear appearance.</p> <p>12-9 Describe the laboratory techniques used to screen for and diagnose thalassemia.</p> <p>12-10 Differentiate thalassemia from iron-deficiency anemia based on laboratory findings.</p> <p>12-11 Identify common treatments for thalassemia, along with their known complications.</p> |
|--|--|

The thalassemias are a diverse group of genetic disorders that clinically manifest as anemia of varying degrees. These disorders are the result of a decrease in production of the globin-chain portion of the hemoglobin molecule. Thalassemias are one of the most common inherited disorders in the world, with an estimated 100,000 to 200,000 infants born with a severe form each year.¹

In 1925, Thomas B. Cooley and Pearl Lee described the first cases of severe thalassemia in several North American children of Mediterranean origin.² "Cooley's anemia" is still a commonly used term for this form of severe thalassemia, which is also termed **thalassemia major**. The name "thalassemia" was

applied to these clinical syndromes a few years later. The term is derived from the Greek word *thalassa*, which means sea because at that time, all of the cases described were from the Mediterranean coastal region. It is now well known that the distribution of thalassemia is worldwide and not restricted to the Mediterranean Sea area. It was later realized that the original severe clinical disease described by Cooley was the result of a homozygous defect in hemoglobin production, whereas many milder cases described as "thalassemia minima" or "thalassemia minor" were manifestations of a heterozygous defect. In contrast with hemoglobinopathies such as sickle cell disease, hemoglobin C, or hemoglobin E, in which the genetic

defect results in a structurally abnormal globin chain (a qualitative change), the thalassemia syndromes result from decreased production of one of the globin chains (a quantitative change). With a few minor exceptions, the globin chains produced are structurally normal, but there is an imbalance in production of the two different types of chain, resulting in an absolute decrease in the amount of normal hemoglobin formed, as well as an excess production of one type of chain that may precipitate and induce hemolysis. There are two major types of thalassemia: alpha (α) thalassemia, which is caused by a defect in the synthesis of α chains; and beta (β) thalassemia, caused by a defect in the synthesis of β chains. The original cases described by Cooley were cases of homozygous β thalassemia.

The world distribution of thalassemia is summarized in Figure 12-1. The thalassemias are predominant in Italy, Greece, West Africa, India, and Southeast Asia. However, due to the movement and migration of individuals, thalassemias are becoming more and more prevalent in areas such as North and South America and Northern Europe.

CRITICAL THINKING QUESTION

12-1 How are the thalassemias different from the hemoglobinopathies?

See answers to all Critical Thinking Questions at the back of this book.

Genetics of Hemoglobin Synthesis

All normal human hemoglobins have the same general tetrameric structure composed of two alpha-like chains and two beta-like chains. The alpha-like chains include alpha (α) and zeta (ζ) chains. The beta-like chains include beta (β), delta (δ), gamma (γ & $\text{G}\gamma$), and epsilon (ϵ) chains. Different types of hemoglobin are produced throughout embryonic, fetal, and adult life. They result from various combinations of

alpha- and beta-like chains. Refer to Table 12-1 for a listing of the composition of the normal human hemoglobins. The table also includes composition of abnormal hemoglobins seen in thalassemia syndromes.

The production of hemoglobin chains occurs from the transcription and translation of genes organized into clusters. The beta gene cluster is located on chromosome 11, while the alpha gene cluster is located on chromosome 16. The structure of the globin genes includes coding regions (**exons**) and noncoding regions (**introns**), along with regulatory sequences found outside the gene clusters. The gene clusters also include **pseudogenes** ($\psi\beta$, $\psi\alpha$, $\psi\zeta$) that are similar in sequence to their normal gene counterparts but do not result in any synthesized globin product.³

The beta-like globin genes on chromosome 11 are arranged in the order that they are produced throughout development. From 5' to 3', their orientation is as follows: $\epsilon - \text{G}\gamma - \text{A}\gamma - \psi\beta - \delta - \beta$. The cluster also includes the Locus Control Region (LCR) that is found on the 5' end of the gene cluster, responsible mainly for gene activation⁴ (Fig. 12-2).

There are two, identical, alpha-like globin genes ($\alpha 1$, $\alpha 2$) found on chromosome 16 that make up a total of four alpha globin genes. However, the $\alpha 2$ gene is expressed at two to three times the rate of the $\alpha 1$ gene.⁵ Each gene is arranged from the 5' to the 3' end as follows: $\zeta - \psi\zeta - \psi\alpha 2 - \psi\alpha 1 - \alpha 2 - \alpha 1$. The alpha gene cluster also includes the regulatory sequence HS40, a nuclear-hypersensitive area that lives upstream from the gene cluster and, like the LCR near the beta cluster, plays an important role in gene activation (Fig. 12-2).

The thalassemia disorders are a direct result of genetic alterations affecting the alpha and beta gene clusters. **Alpha thalassemias** occur mainly due to gene deletions, whereas **beta thalassemias** result from various genetic mutations.^{6,7} Genetic mutations that lead to thalassemias most often include those of the promotor sequences, nonsense mutations, stop codons, and splice site mutations. They influence globin chain

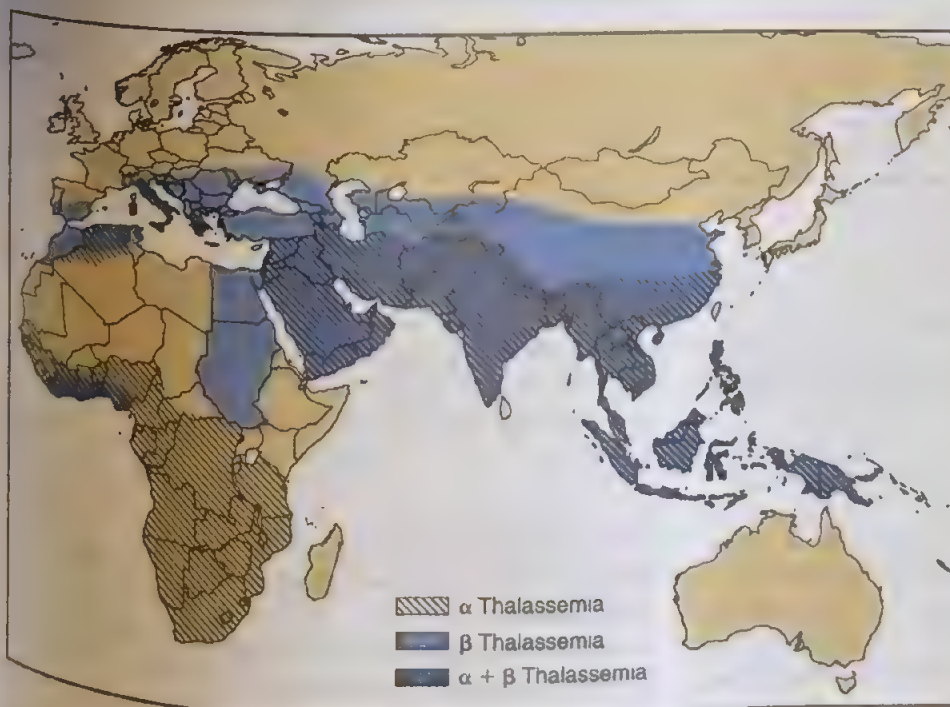


FIGURE 12-1 World distribution of alpha (α) and beta (β) thalassemia.

TABLE 12-1 Composition of Hemoglobins Found in Normal Human Development and Abnormal Hemoglobins Found in Thalassemia

State	Globin Chains	Hemoglobin
Adult	$\alpha_2\beta_2$	A
	$\alpha_2\delta_2$	A ₂
Fetus	$\alpha^A\gamma_2$	F
	$\alpha^G\gamma_2$	F
Embryo	$\alpha_2\varepsilon_2$	Gower 2
	$\zeta_2\varepsilon_2$	Gower 1
	ζ_{292}	Portland
α Thalassemia	β_4	H
	γ_4	Bart's

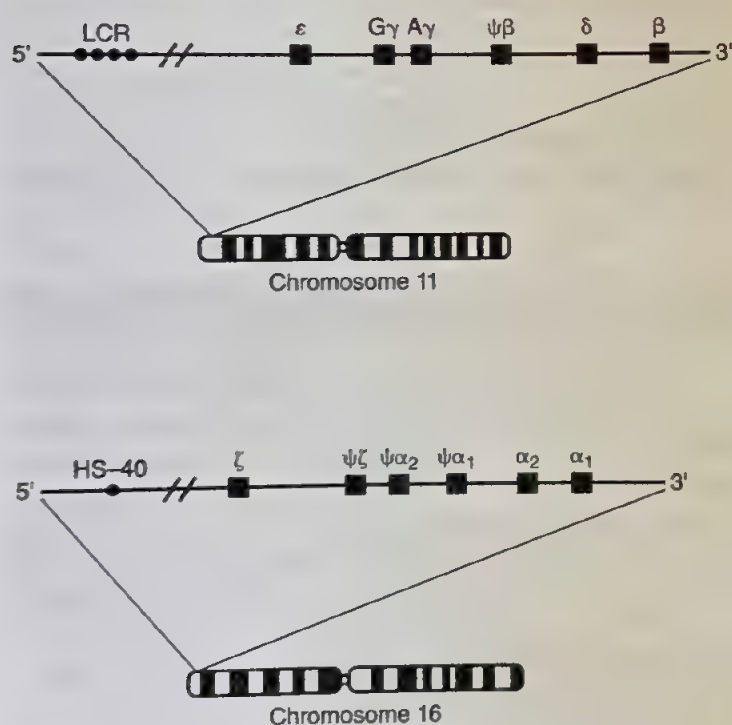


FIGURE 12-2 Top: Diagram of the β gene cluster on chromosome 11 including the locus control region (LCR) at the 5' end. Bottom: Diagram of the α gene cluster on chromosome 16 including the nuclear-hypersensitive area at the 5' end.

production by interfering at various stages of transcription, RNA processing, and translation.⁸ However, in all cases, the result is the same: decreased or absent production of globin chains.

Pathophysiology

The pathology of thalassemia results from an imbalance of globin chains. Decreased production of a single globin chain leads to an overall decrease in normal functional hemoglobin resulting in a microcytic, hypochromic anemia. Because α -like and β -like globin chains assemble in a balanced fashion, decreased production of one globin chain results in a

relative excess of the other globin chain. For example, in β thalassemia, there is a reduced or absent β chain production resulting in an excess of unmatched α globin, which then accumulates in red blood cells. The accumulation of excess globin chains damages the red blood cell or red blood cell precursors, manifesting as either peripheral hemolysis or ineffective erythropoiesis, respectively.

In the case of α thalassemia, the excess γ chains and β chains can form tetramers: hemoglobin Bart's (γ_4) and hemoglobin H (β_4), respectively. However, these hemoglobins are physiologically useless and will precipitate in older red blood cells, causing a shortened red cell life span. These abnormal hemoglobins may be detected in the peripheral blood as a diagnostic clue for α thalassemia. In the case of β thalassemia, the excess α chains form α_2 precipitates that trigger apoptosis of the red cell precursors in the bone marrow, resulting in ineffective erythropoiesis. In severe forms of β thalassemia, there is a selective survival of cells producing hemoglobin F, which then becomes a diagnostic clue for a homozygous form of β thalassemia. Although both are ineffective, erythropoiesis and hemolysis play roles in α and β thalassemia syndromes; hemolysis predominates in severe α thalassemia, whereas ineffective erythropoiesis predominates in severe β thalassemia (Fig. 12-3).

CRITICAL THINKING QUESTION

12-2 How could a lab scientist working in the hematology department detect thalassemia in a patient simply through peripheral blood smear analysis?

Thalassemia Syndromes

A Broad Clinical Classification of Thalassemia Syndromes

Because of the heterogeneity of thalassemia at the molecular level, it may be helpful to broadly categorize the thalassemia syndromes according to clinical severity. The clinical con-

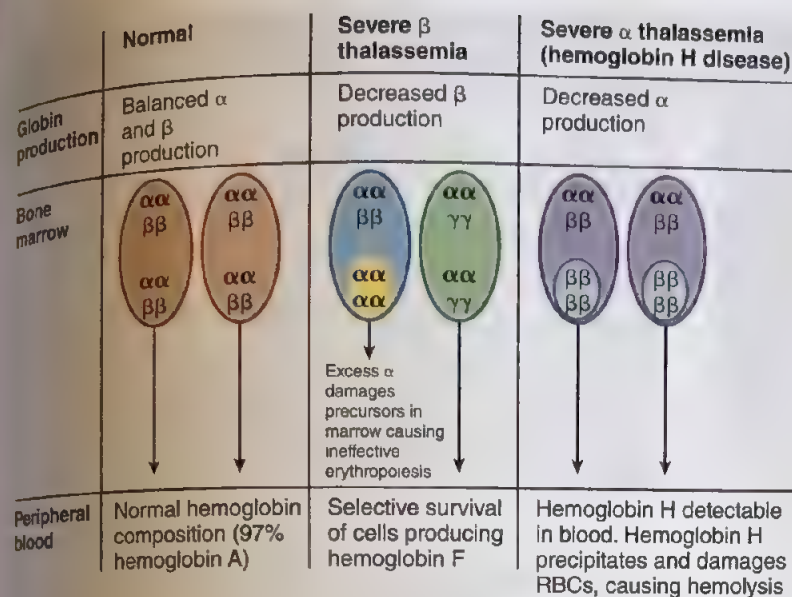


FIGURE 12-3 Diagram of globin chain imbalance. Excess globin chains precipitate and damage red blood cells and their precursors. Destruction of red blood cells within the bone marrow (ineffective erythropoiesis) predominates in severe β thalassemia. In contrast, hemoglobin H precipitates and damages circulating red blood cells (hemolysis) in severe α thalassemia. Notice the changes in hemoglobin constitution of the peripheral blood with thalassemia: hemoglobin F is increased in severe β thalassemia and hemoglobin H is detectable in severe α thalassemia.

and therapy of patients with thalassemia can be broadly subdivided into three categories: thalassemia major, thalassemia intermedia, and thalassemia minor. A fourth category, termed thalassemia minima, is applied to healthy silent carriers who show no clinical symptoms and minimal to no hematologic abnormalities. The different genetic backgrounds that result in each of these clinical outcomes are summarized in Table 12-2.

Beta Thalassemia

The genetic basis of β thalassemia is extremely heterogeneous and results from the inheritance of various combinations of genetic mutations. Almost 400 different mutations have been described to date.^{10,11} However, only about 20 mutations account for around 80% of all β -thalassemia genes worldwide. A specific group of mutations (four to six) is characteristically found in each geographic area. The more severe forms of β thalassemias are most prevalent in patients from Italy, Greece, Algeria, Saudi Arabia, and Southeast Asia.¹²

Beta Thalassemia Genetic Nomenclature

Clinically, groups of inherited genetic mutations may be broadly subdivided and designated by the gene symbols β^0 or β^+ . The β^0 gene symbol refers to a set of genetic mutations that result in the complete absence of β chains. The β^+ gene symbol refers to a set of genetic mutations that result in reduced amounts of β globin. The wide variety of mutations at the molecular level produces a spectrum of β^+ thalassemia genes, expressing various levels of β globin. The expression of β globin is inversely proportional to the severity of clinical presentation. In other words, mutations causing large decreases in β globin will result in severe clinical syndromes, whereas mutations causing only small decreases in β globin will result in mild, or even silent, clinical syndromes.

Because of the heterogeneity of the genetic background of beta thalassemia, the resulting phenotypes of inherited genes do not always present as the same disorder. For example, two individuals that inherit the same beta thalassemia mutations may not express identical phenotypes. For this reason, it is clinically more useful to refer to the phenotypic categories of

the beta thalassemias rather than their genotypes. The phenotypic categories include β thalassemia major, β thalassemia intermedia, and β thalassemia minor. Each phenotypic category varies in clinical course including onset, clinical presentation, and survival.

Beta Thalassemia Major

β Thalassemia major is the most severe clinical expression of β thalassemia and occurs in patients with homozygous β^0 or β^+ thalassemia (β^0/β^0 , β^+/β^+) or with compound heterozygous β^0 and β^+ thalassemia (β^0/β^+). Homozygous β thalassemias are encountered in geographic regions with greater consanguinity. However, it is more common for a β thalassemia major patient to have a compound heterozygous disorder. When two inherited mutations are severe, they greatly decrease the production of β globin, producing severe, transfusion-dependent clinical disease.

β Thalassemia does not manifest in utero because the fetus produces fetal hemoglobin, hemoglobin F ($\alpha_2\gamma_2$), rather than adult hemoglobin, hemoglobin A ($\alpha_2\beta_2$). The transition, or switch, from fetal to adult hemoglobin begins in the third trimester and continues until complete at about 6 months; therefore, infants with β thalassemia syndromes do not present clinically until after birth.

In thalassemia major, a severe hypochromic, microcytic anemia develops during the first year of life. The hemoglobin level is typically <7 g/dL and consists mostly of hemoglobin F and hemoglobin A₂.¹¹ Infants with β thalassemia major usually present within the first year of life with failure to thrive, pallor, recurrent infections, and abdominal enlargement due to splenomegaly. On clinical presentation, the diagnosis can be confirmed with the presence of anemia, abnormal blood smear (see Fig. 12-4), elevated hemoglobin F, and the demonstration of the β thalassemia trait in both parents.

In nontransfused and inadequately transfused infants, this severe chronic anemia is a strong stimulus for erythropoiesis. This causes marked expansion of the marrow space and characteristic skeletal changes of the skull, long bones, and hand bones. The skull radiographs show widening of

TABLE 12-2 Genetic Background of the Different Clinical Courses of Thalassemia

Genetic Background	Clinical Syndrome
Homozygotes	
Homozygous β^0	Thalassemia major
Homozygous β^+	Thalassemia major or intermedia
Homozygous $\delta\beta$	Thalassemia intermedia
Homozygous Hb Lepore	Thalassemia major or intermedia
Homozygous HPFH	Thalassemia minima
Homozygous α^0 -thalassemia ($-/-$)	Hemoglobin Bart's hydrops fetalis
Homozygous α^+ -thalassemia ($-\alpha/-\alpha$)	Thalassemia minor
Compound Heterozygotes	
Compound heterozygous β^0 /severe β^+	Thalassemia major
Compound heterozygous β^{++} /severe β^+ or β^0	Thalassemia major or intermedia
Compound heterozygous $\delta\beta/\beta^0$	Thalassemia major or intermedia
Compound heterozygous Lepore/ $\delta\beta$	Thalassemia intermedia
Compound heterozygous Lepore/ β^0 or β^+	Thalassemia major
Compound heterozygous HPFH/ β^0 or β^+	Thalassemia minor
Compound heterozygous E/ β^0 or β^+	Thalassemia major or intermedia
Compound heterozygous C/ β^0 or β^+	Thalassemia intermedia or minor
Compound heterozygous S/ β^0 or β^+	Sickle cell disease
Compound heterozygous α -thalassemia ($-/-\alpha$)	Thalassemia intermedia (hemoglobin H disease)
Heterozygotes	
Heterozygous β^0 or β^+	Thalassemia minor
Heterozygous β^{++}	Thalassemia minima
Heterozygous $\delta\beta$	Thalassemia minor
Heterozygous Lepore	Thalassemia minor
Heterozygous HPFH	Thalassemia minima
Heterozygous α^0 thalassemia	Thalassemia minor
Heterozygous α^+ thalassemia	Thalassemia minima

the diploid space and characteristic radiating striations giving the typical "hair-on-end" appearance (Fig. 12-5). The marrow expansion of the facial bones produces a characteristic facial appearance with hypertrophy of the maxilla causing forward protrusion of the upper teeth and overbite, a relatively sunken nose, widely spaced eyes, and prominent cheek bones, resulting in what is referred to as craniofacial abnormalities (Fig. 12-6). The long bones of the hands and feet have cortical thinning with porosity of the medullary space on radiographs. These changes are not a specific feature of β thalassemia and are found in other severe, chronic congenital anemias, but they are most prominent in β thalassemia major.

Without careful medical supervision and a therapeutic program (see later), these children will have numerous

complications, including massive hepatosplenomegaly, recurrent infections, spontaneous fractures, leg ulcers, dental and orthodontic problems, and compression syndromes caused by tumor masses from extramedullary hematopoiesis. If the condition is left untreated, these children will usually die in early childhood.

Beta Thalassemia Intermedia

β Thalassemia intermedia is a less severe clinical expression and occurs in patients homozygous or heterozygous for combinations of less severe β -globin mutations ($\beta^+\beta^+$, $\beta^0\beta^+$). β Thalassemia intermedia covers a broad spectrum of clinical expression of thalassemia, bridging the gap between severe β thalassemia major and the mild, often asymptomatic anemic state of β thalassemia minor. The definition



FIGURE 12-4 Peripheral smear from a patient with β -thalassemia major. Note the nucleated red cells, Howell-Jolly body in the hypochromic microcyte (arrow), numerous target cells, and moderate anisocytosis and poikilocytosis (Wright's stain). (From Bell A. Hematology. In: Listen, Look and Learn. Bethesda, MD: Health and Education Resources, Inc., with permission.)



FIGURE 12-5 Skull x-ray film of a 5-year-old child with homozygous β -thalassemia. Note the dilation of the diploic space and the typical "hair-on-end" appearance caused by subperiosteal bone growth in the diploic space.

β -thalassemia intermedia is relative because the clinical state varies from mild to severe.

Patients with β -thalassemia intermedia usually present at a somewhat older age (generally after the age of 2) and with a slightly higher level of hemoglobin (7 to 10 g/dL) than patients with β -thalassemia major. Patients demonstrate variable degrees of symptomatic anemia, jaundice, splenomegaly, and survive into adulthood without a large blood transfusion requirement.

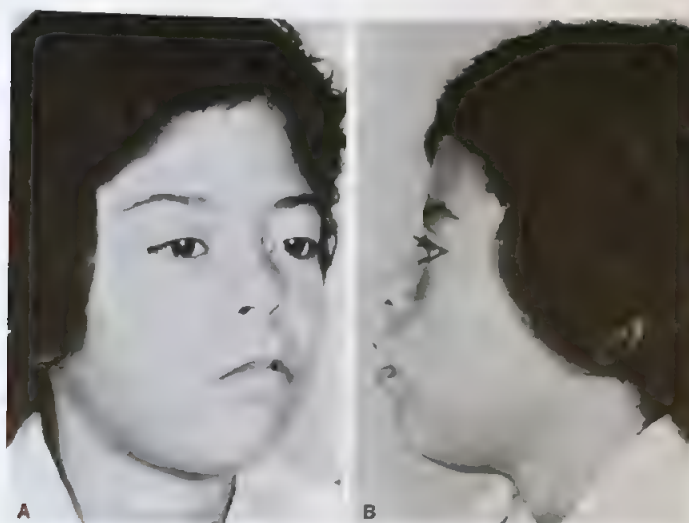


FIGURE 12-6 Face (A) and profile (B) of an 11-year-old child, with homozygous β -thalassemia who is receiving hypertransfusion. The characteristic craniofacial changes are not as prominent as those in an untransfused child but are still present. Note the bossing of the skull, hypertrophy of the maxilla with prominent malar eminences, depression of the bridge of the nose, and slant of the eyes.

The serum bilirubin level is significantly more elevated in patients with β -thalassemia intermedia than in those with β -thalassemia major. This is a result of dysregulated hepcidin in β -thalassemia minor patients, which leads to increased iron absorption in the gastrointestinal tract and subsequent iron overload in the liver.¹³ These patients may develop the physical and bony characteristics of β -thalassemia major including hepatosplenomegaly. The anemia usually becomes worse with infections, pregnancy, or folic acid deficiency states. These patients are susceptible to frequent, sometimes severe, infections and gallbladder problems owing to the formation of gallstones. Children usually have an acceptable level of growth and development (although puberty may be delayed by a few years), and they reach adulthood if infections are controlled and if they enjoy good nutrition with particular emphasis on prevention of folic acid deficiency. They may become transfusion dependent if severe hypersplenism occurs. This usually requires splenectomy. Women with β -thalassemia intermedia may become pregnant and may require blood transfusions as well as folic acid supplementation throughout pregnancy. In spite of the lack of transfusion, patients with β -thalassemia intermedia develop iron overload as a result of the increased absorption.¹⁴

Beta Thalassemia Minor

β -Thalassemia minor is the least severe clinical form of beta thalassemia and occurs from the heterozygous inheritance of either the β^0 or β^+ thalassemia gene (β^0/β , β^+/β). Patients with thalassemia minor are usually diagnosed incidentally to a family study of an index case with thalassemia major, by population screening, or by an incidental laboratory finding. Laboratory findings include a mild microcytic, hypochromic anemia, usually in the 10 to 13 g/dL range (Fig. 12-7). In general, the levels of hemoglobin A₂ and F are mildly elevated. Patients are asymptomatic except during periods of stress such as pregnancy, infection, or folic acid deficiency.

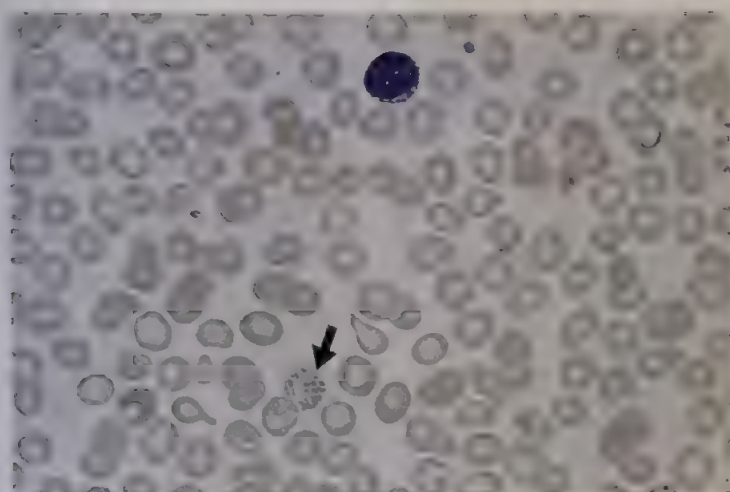


FIGURE 12-7 Peripheral smear from a patient with thalassemia minor. Note the microcytosis and hypochromia with mild anisocytosis and poikilocytosis. A few target cells and basophilic stippling are present. (Wright's stain, magnification $\times 400$)

Some patients with very mild mutations show no clinical or laboratory evidence of anemia (normal hemoglobin A_2). They are sometimes designated by the β^{++} haplotype and are called *silent carriers*. Beta thalassemia minor patients usually require no therapy if they maintain good nutrition and exhibit normal survival rates. However, it is important that they not be misdiagnosed as having iron deficiency, since iron deficiency anemia requires specific treatment.

CRITICAL THINKING QUESTION

12-3 Why is there such heterogeneity in the clinical presentation of the beta thalassemias?

Alpha Thalassemia

Remember α thalassemias result mainly from gene deletions in which one or more of the alpha genes is rendered non-functional, rather than the genetic mutations seen in the beta thalassemias that lead to varying levels of β globin production. There are also less common, nondeletional causes of α thalassemias and certain mutations that result in unstable α globin chains. The nondeletional α thalassemias tend to be more severe. However, despite the genetic defect, the result is a decrease in alpha-globin production. Alpha thalassemias are most common in patients from the regions of the Mediterranean, Africa, the Middle East, India, and all of East and Southeast Asia. The most severe cases occur in Southeast Asia and certain Mediterranean Islands.¹⁵

Alpha Thalassemia Genetic Nomenclature

The α^0 gene designation, also sometime called alpha thalassemia 1, refers to a deletion of both α genes ($\alpha 1$ and $\alpha 2$) on the same chromosome. As a consequence, there is a completely absent production of α chains from the affected chromosome. Because both α -globin genes are deleted, the genotype can be designated $--$, and a patient who is homozygous for the α^0 thalassemia mutation would be designated $--/--$.

The typical α^+ thalassemia genotype, also referred to as alpha thalassemia 2, has a deletion of a single α -globin gene

on chromosome 16, which leaves the other α -globin gene intact and able to function. Deletion of one α -globin gene is characterized by a reduced output of α chains. This deletion mutation is also denoted as $-\alpha$. Other less common types of α^+ thalassemia genes are caused by nondeletion mutants, $\alpha^{ND}\alpha$, affecting α chain synthesis. This is similar to the situation in the β thalassemias in which numerous different mutations can occur. Depending on the type and location of the mutation, several processes can be affected that lead to decreased production of α globin. A third type of α^+ thalassemia genetic background is associated with highly unstable α -globin structural mutants, which precipitate in red blood cells. An example is hemoglobin Constant Spring, resulting in a long and unstable alpha chain.

Unlike the β thalassemias, the α -thalassemia haplotypes correlate fairly well with their respective phenotypes. The phenotypes can be classified into four categories: α thalassemia major (hemoglobin Bart's hydrops fetalis), hemoglobin H disease, α thalassemia minor, and the α thalassemia silent carrier. Each phenotypic category varies in clinical course including onset, clinical presentation, and survival, which will be discussed in the following sections.

In contrast to β thalassemia, α thalassemia is usually manifested immediately at birth or in utero. This early presentation reflects the switch from embryonic hemoglobin to fetal hemoglobin ($\alpha_2\gamma_2$) around 6 to 8 weeks of gestation.

Alpha Thalassemia Major (Bart's Hydrops Fetalis)

The most severe expression of α thalassemia is α thalassemia major, also known as hemoglobin Bart's hydrops fetalis, which is caused by homozygous inheritance of the α^0 thalassemia gene ($--/--$). This is a lethal disease, and infants with hemoglobin Bart's hydrops fetalis die either in utero or soon after birth. They produce no α chains, and the only hemoglobins found are hemoglobin Bart's (γ_4) and hemoglobin Portland ($\zeta_2\gamma_2$). Because hemoglobin Bart's is useless as an oxygen carrier, survival of these fetuses into the third trimester or until birth is entirely due to the presence of hemoglobin Portland. This condition is quite common in Southeast Asia but is also found sporadically in the Mediterranean area.

At delivery, these infants are severely anemic and edematous, and demonstrate ascites, marked hepatomegaly, and splenomegaly. The mothers may report a history of stillbirth or neonatal deaths. The clinical significance of this entity is related to the obstetric problems that may arise in the affected infants' mothers. Pregnancy is often complicated by pre-eclampsia, labor complications, and postpartum hemorrhage, all of which contribute to severe morbidity and mortality. Clinical emphasis for this entity is on the prevention of the disease through early antenatal diagnosis. In the hands of skilled clinicians, ultrasound screening may be used to identify Bart's hydrops fetalis as early as the first trimester. In countries that have the means, it is more commonly diagnosed with chorionic villus sampling.¹⁶

Hemoglobin H Disease

The second most severe clinical expression of α thalassemia is hemoglobin H disease. In this entity, only one α -globin gene out of four is functional. This is usually the result of

a compound heterozygosity of the deletional α^0 thalassemia and α^+ -thalassemia haplotypes ($-/-\alpha$) but can also arise from individuals who have nondeletion mutations ($-/\alpha^{ND}\alpha$ or $\alpha^{ND}\alpha/\alpha^{ND}\alpha$). Nondeletional hemoglobin H disease usually results in a more severe clinical course.¹⁷

Clinically, hemoglobin H disease is similar to β thalassemia intermedia in that it covers a wide range of severity. Patients vary from having a mild anemia to potentially requiring regular transfusion. Because there is a switch from fetal (γ) to adult (β) globins around the time of birth, infants and adults have different hemoglobin compositions. Adults with hemoglobin H disease will have from 5% to 40% hemoglobin H; the remainder is mostly hemoglobin A with a small amount of hemoglobin A₂ and hemoglobin Bart's. Infants who later develop hemoglobin H disease usually have between 19% and 27% hemoglobin Bart's at birth, with the remainder composed of hemoglobin F and hemoglobin A. Hemoglobin H and hemoglobin Bart's can easily be identified by hemoglobin electrophoresis, because they migrate anodal to hemoglobin A at alkaline pH (Fig. 12-8). These are sometimes referred to as fast hemoglobins. In addition, hemoglobin H shows a characteristic appearance of multiple ragged inclusions in many red cells after incubation with brilliant cresyl blue, the so-called golf ball appearance (Fig. 12-9).

Because infants have nearly normal hemoglobin levels and no splenomegaly, the diagnosis must come from clinical suspicion and laboratory testing. Most cases are discovered in adulthood and will have episodic periods of anemia, pallor, and weakness during which they may be discovered to have a hypochromic, microcytic anemia, leading to an eventual diagnosis. These episodes of anemia may be associated with concurrent infections, medications, or other illnesses. Other clinical features may include scleral icterus, hepatosplenomegaly, gallstones, and bone marrow expansion. The bone changes are present in about one-third of cases but are milder than that seen in severe β thalassemia. Splenomegaly, if present, may worsen the anemia and sequester platelets, so these patients may benefit from a splenectomy. Some cases of hypercoagulability, with deep vein thrombosis and pulmonary embolism, have been reported after splenectomy. Unlike severe β thalassemia, iron overloading is not a general feature

of hemoglobin H disease but may occur in older patients and patients requiring regular transfusion. Anemia may worsen during pregnancy, and when it occurs, other factors such as iron or folate deficiency should be investigated. Patients over 6 years old can be considered to have hemoglobin H disease if greater than 1% to 2% hemoglobin H is detected, and there are typical inclusion bodies in the red blood cells after incubation with brilliant cresyl blue.

Alpha Thalassemia Minor

α Thalassemia minor is most commonly caused by defects of two of the four α -globin genes. This is usually the result of heterozygous inheritance of α^0 thalassemia genes ($-/-\alpha$) but could also be the result of homozygous inheritance of α^+ thalassemia genes ($-\alpha/-\alpha$). The condition is characterized by the presence at birth of 5% to 15% hemoglobin Bart's, which disappears with development and is not replaced by hemoglobin H. Adults, therefore, will have a normal hemoglobin electrophoretic pattern.

Patients with α thalassemia minor are generally asymptomatic with mild microcytic hypochromic anemia. The mean corpuscular volume (MCV) is usually between 70 and 75 fL. They are identified either incidentally or by screening programs in regions with a high prevalence of α thalassemia. This condition exists in 3% of African Americans and may be confused with iron deficiency. Identification of individuals with α thalassemia minor is, however, very important. Pregnant women with α thalassemia minor and women with α thalassemia minor who are planning pregnancies are at risk for hemoglobin Bart's hydrops fetalis and its associated morbidity and mortality. Identification of thalassemia minor is also important to prevent the unnecessary treatment of misdiagnosed iron deficiency.

Alpha Thalassemia Silent Carrier

The last category of α thalassemia is the *silent carrier of α thalassemia*, due to the inheritance of one α^+ thalassemia allele (also called alpha thalassemia 2 allele). This is the result of a defect in one of the four α -globin genes and is characterized by the presence of a very small amount (up to 2%) of hemoglobin Bart's at birth. After the disappearance of hemoglobin Bart's during development, no recognizable hematologic abnormality

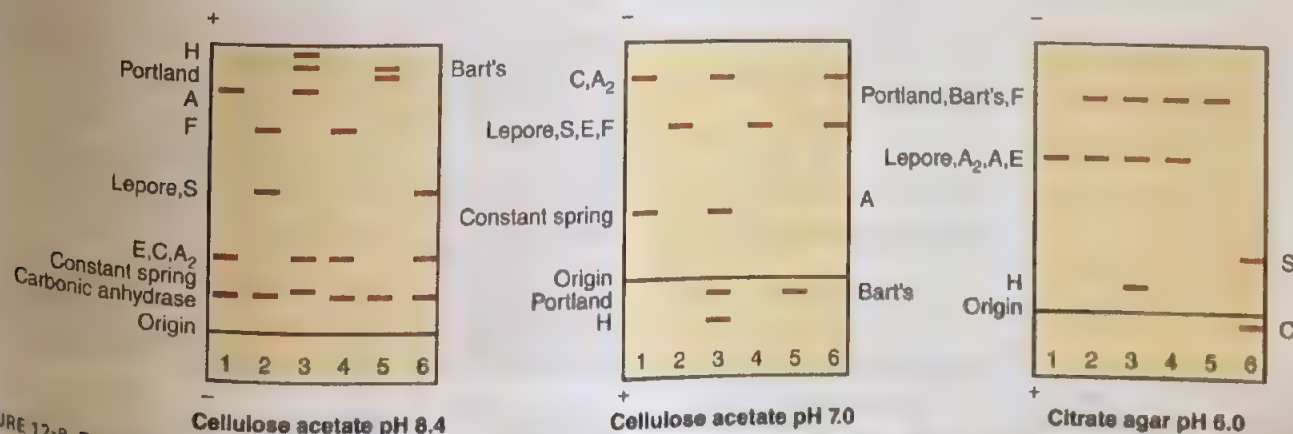


FIGURE 12-8 Diagram of the migration of the different hemoglobins at different pH: (1) normal adult (A, A₂); (2) homozygous Hb Lepore (F, Lepore); (3) HbH/Constant Spring disease $\alpha\alpha/\alpha\alpha^{CS}$ (Constant Spring, A, A, Bart's H); (4) compound heterozygous HbE/ β -thalassemia (E, F); (5) Hb Bart's hydrops fetalis syndrome (Bart's, H); (6) Hb Portland (Portland, A, A₂, F, Lepore, S, E, C, A₂, Constant spring, Carbonic anhydrase, Origin).

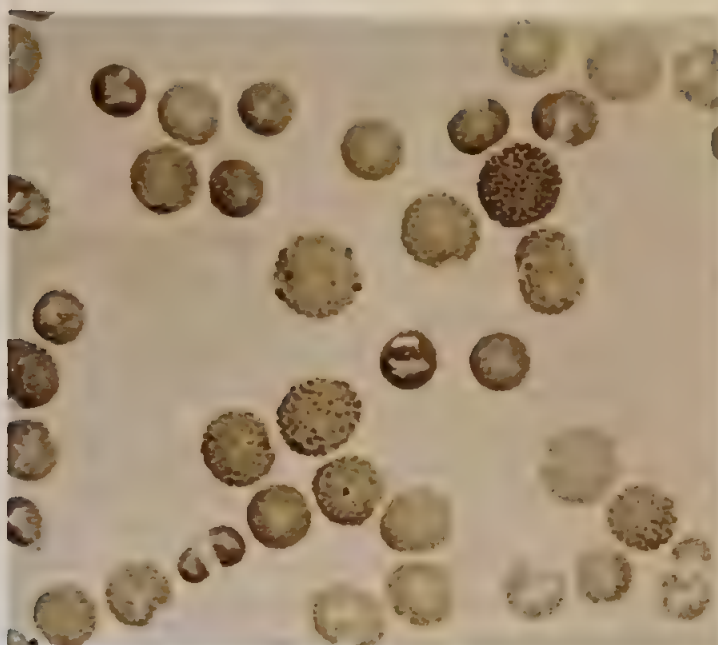


FIGURE 12-9 Hemoglobin H inclusions (supravital stain). (From Bell A. Hematology. In: Listen, Look and Learn. Bethesda, MD: Health and Education Resources, Inc., with permission.)

is present, except for a borderline low MCV (78 to 80 fL). This condition is found in up to 30% of African Americans.

Patients who carry the α^+ thalassemia haplotype are asymptomatic and, again, may only be picked up by screening programs or by incidental, abnormal laboratory data. These individuals are not anemic and have normal blood smears, but a slightly low MCV can be a clue to the carrier state. Women who are silent carriers are not at risk of hemoglobin Bart's hydrops fetalis; however, they may have children with hemoglobin H disease.

There is a need to separate α thalassemia minor from silent carrier of α^+ thalassemia in women because of the risk associated with hemoglobin Bart's hydrops fetalis. Although the level of hemoglobin Bart's at the time of birth may be predictive of α thalassemia minor versus silent carrier, there is overlap. Also, ethnicity may be helpful when estimating risk. The only way to know for sure is through molecular analysis of the α genes.

In the simplest terms, deletional type mutations result in predictable clinical syndromes such that four deletions produce hemoglobin Bart's hydrops fetalis, three deletions produce hemoglobin H disease, two deletions produce α thalassemia minor, and one deletion produces a silent carrier (Fig. 12-10). Because nondeletion mutations and hemoglobin variants can also combine with deletional α -globin gene mutations, the actual molecular basis for α thalassemia syndromes is more heterogeneous. The different genetic backgrounds associated with the four different clinical expressions of α thalassemia are summarized in Table 12-3.

Other Thalassemias and Thalassemia-Like Conditions

Hemoglobin Constant Spring

One unique and important nondeletional α -globin variant is hemoglobin Constant Spring. The mutation is abbreviated as α^{CS} and is the result of a point mutation in the α_2 -globin gene. It may be considered an α^+ thalassemia genotype because it affects only one of the two α -globin genes. The point mutation alters the natural stop codon, allowing translation to continue until the next stop codon farther downstream on the mRNA. The end result is an α -globin variant with 31 extra amino acids at its tail end. α^{CS} combines with β -globin to produce hemoglobin Constant Spring (Hb CS). Hb CS can be detected on hemoglobin electrophoresis at alkaline pH as a slow migrating band. Heterozygous patients are asymptomatic without hematologic abnormalities and produce only small amounts of Hb CS (1%), while homozygotes have α thalassemia minor with microcytosis and 5% to 8% Hb CS. Hb CS is found in Southeast Asia, where it can combine with other prevalent α thalassemia haplotypes to produce α thalassemia syndromes.

Hereditary Persistence of Fetal Hemoglobin

Hereditary persistence of fetal hemoglobin (HPFH) comprises a group of conditions characterized by the persistence of fetal hemoglobin synthesis into adult life. HPFH is caused by either an absence of δ - and β -chain synthesis or a lack of γ -chain suppression. Depending on the genetic defect, this can result in a complete absence of HbA and HbA₂, leaving all hemoglobin produced in the adult to be Hb F. However, this large production of Hb F compensates for the loss of δ - and

FIGURE 12-10 Simplistic look at the interactions of deletional mutations of α thalassemia. The severity of disease is predictable and depends on the number of deleted α -globin genes.

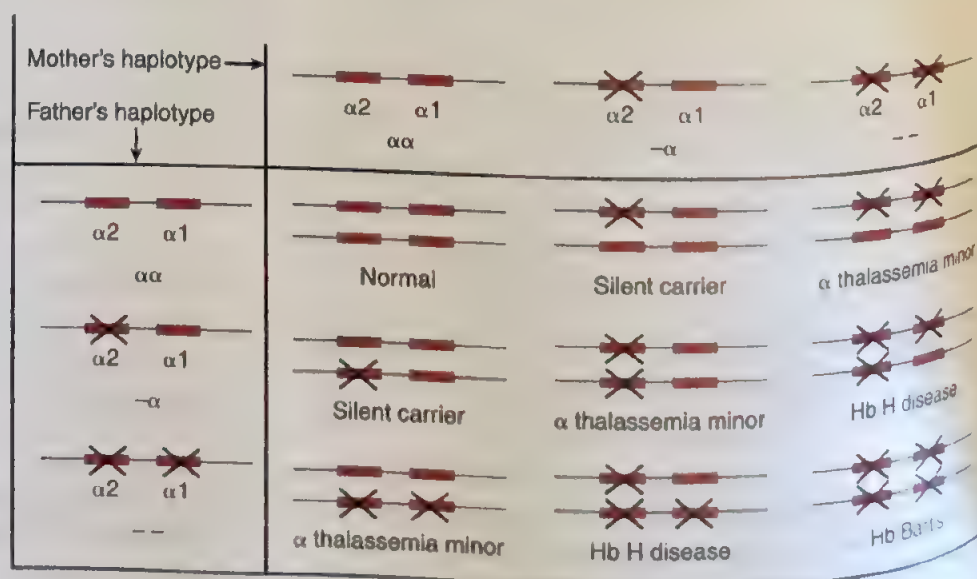


TABLE 12-3 Genetic Background of α Thalassemia Clinical Syndromes (Mating Combinations)

Genes $\downarrow \rightarrow$	$\alpha\alpha$	$-\alpha$	$\alpha^{\text{CS}}\alpha$	$\alpha^{\text{Hb}}\alpha$	--
$\alpha\alpha$	N	α carrier	α carrier	α carrier	thal minor
$-\alpha$	α carrier	thal minor	thal minor	thal minor	H
$\alpha^{\text{CS}}\alpha$	α carrier	thal minor	thal minor	thal minor	H
$\alpha^{\text{Hb}}\alpha$	α carrier	thal minor	thal minor	H	H
--	thal minor	H	H	H	Bart's

$\alpha\alpha$ = normal genotype; $-\alpha$ = deletion of one α -globin gene; $\alpha^{\text{CS}}\alpha$ = Hb Constant Spring; $\alpha^{\text{Hb}}\alpha$ = nondeletion α thalassemia gene; -- = deletion of both α -globin genes (Note: the clinical phenotype resulting from the combination of these genotypes is found at the intersection of the corresponding column and row); N = normal clinical phenotype; α carrier = silent carrier of α thalassemia, 5%–15% Hb Bart's at birth, mild anemia; thal = α thalassemia minor, 0%–2% Hb Bart's at birth, minimal hematologic changes; H = hemoglobin H disease; Bart's = hemoglobin Bart's hydrops fetalis.

β -globin chains, so patients with HPFH do not experience significant hematologic abnormalities.

There are two categories of genetic defects causing HPFH. The first is a deletion within the beta globin gene cluster, and the other includes mutations or polymorphisms of regulatory genes such as the promotor of the γ -globin gene.¹⁸ HPFH can also be classified into two categories according to the distribution of hemoglobin F among the red cells. Fetal hemoglobin is more resistant than adult hemoglobin to elution at acid pH and can be demonstrated on a peripheral smear by the acid elution test of Kleihauer and Betke. Using this stain, the HPFH conditions can be divided into a pancellular form, in which hemoglobin F is uniformly distributed among the red cells, and a heterocellular form, in which hemoglobin F is found in only a small percentage of the cells (Fig. 12–11). In a normal adult, cells containing hemoglobin F, also called F cells, can occasionally be found, but the amount is always less than 2% and is usually less than 1%.

More recently, flow cytometry has been used to separate pancellular and heterocellular distributions of hemoglobin F. This technique is discussed later in this chapter with the other laboratory methods useful in the diagnosis of thalassemia.



FIGURE 12-11 Kleihauer-Betke stain of blood from a patient with hereditary persistence of fetal hemoglobin (HPFH). Note that all red cells stain red, owing to the varying amounts of hemoglobin F. (From Bell A. Hematology In: Listen, Look and Learn. Bethesda, MD: Health and Education Resources, Inc., with permission.)

Pancellular HPFH Various forms of $(\delta\beta)^0$ HPFH have been described, all of which produce a pancellular distribution of hemoglobin F. One common form is the African (HPFH 1), in which there is a deletion of the δ - and β -globin genes and increased synthesis of $\alpha\gamma$ and $\beta\gamma$ chains, which almost completely compensate for the lack of production of δ and β chains. Hemoglobin F constitutes 100% of the hemoglobin in the homozygous state and 15% to 30% of the hemoglobin in the heterozygous state. The hemoglobin F is homogeneously distributed among the red cells and consists of a mixture of $\alpha\gamma$ and $\beta\gamma$ chains. Clinically, the homozygotes will demonstrate features of thalassemia minor and the heterozygotes will be hematologically normal.

Nondeletion HPFH, $\alpha\gamma\beta^+$ and $\beta\gamma\beta^+$, generally produces a pancellular distribution of hemoglobin F. An example is the Greek/Sardinian/African $\beta\gamma\beta^+$ HPFH, in which about 15% hemoglobin F is present in the heterozygous state. This hemoglobin F is also found uniformly distributed among the red cells but is only of the $\beta\gamma$ type. The homozygous state for this type of pancellular HPFH produces no significant hematologic abnormalities.

Heterocellular HPFH Heterocellular HPFH appears to be an inherited condition in which the number of F cells is increased without concurrent alteration in δ - and β -chain production. Collectively these are called the heterocellular HPFH, or sometimes "Swiss HPFH," but in reality, they are a heterogeneous group, which has not been completely elucidated at the molecular level. These generally produce a mild increase in hemoglobin F, which is rarely greater than 5%. Some of these are the result of a point mutation and rearrangements, while others are not even linked to the β -globin gene cluster.

ADVANCED CONTENT

Delta-Beta Thalassemia and Hemoglobin Lepore Syndrome

Delta-beta ($\delta\beta$) thalassemias are a diverse group of thalassemias characterized by a combined defect in δ - and β -chain synthesis. They can be described as demonstrating a near-normal level of hemoglobin A₂ and an unusually

high level of hemoglobin F in the heterozygote, and absent hemoglobin A and A₂ in the homozygote. All $\delta\beta$ thalassemias studied thus far have been shown to be the result of a deletion. They can be described at the genetic level as three different entities, depending on the amount of DNA lost: hemoglobin Lepore syndrome ($\delta\beta$)⁺ results from a partial deletion of the δ - and β -globin genes, ($\delta\beta$)⁰ thalassemia from a complete deletion of the δ - and β -globin genes, and ($\Delta\gamma\delta\beta$)⁰ thalassemia from a deletion of the $\Delta\gamma$ -globin gene in addition to the deletion of the δ - and β -globin genes. $\delta\beta$ Thalassemias are less common than β thalassemias and have been found sporadically in Greeks, Africans, Italians, and Arabs.

Historically, $\delta\beta$ thalassemias were divided into two groups according to the types of fetal hemoglobin produced. To be more consistent with nomenclature of other thalassemias, the $\delta\beta$ thalassemias are now designated according to the deficiency of globin chains: ($\delta\beta$)⁺, ($\delta\beta$)⁰, and ($\Delta\gamma\delta\beta$)⁰.

One particular type of $\delta\beta$ thalassemia involves the production of an abnormal hemoglobin called hemoglobin Lepore, named after the family in which it was first found. It has been shown to be a fusion of the δ and β chains, which is the product of a fusion gene formed by an unequal crossing over. At least three different hemoglobin Lepores have been described, varying in the exact location of the unequal crossing over. Heterozygosity for $\delta\beta$ thalassemia and hemoglobin Lepore results in a mild form of anemia that is clinically described as thalassemia minor and is similar to the condition of patients with heterozygous β thalassemia.

The γ -chain synthesis in $\delta\beta$ thalassemia is usually more efficient than in β thalassemia or hemoglobin Lepore, and in general, $\delta\beta$ thalassemia produces a milder clinical disease than the latter two. The γ chain is able to combine effectively with α chain to make functional hemoglobin (hemoglobin F); therefore, the accumulation of unmatched α chain, which can damage cells, is reduced. The overall effect is a partial compensation by hemoglobin F for the decreased production of hemoglobin A and A₂. Patients with homozygous $\delta\beta$ thalassemia have a clinical course similar to β thalassemia intermedia. On the other hand, patients with the homozygous state for hemoglobin Lepore, which does not compensate as well as $\delta\beta$ thalassemia, have a more severe transfusion-dependent thalassemia.

Patients with $\delta\beta$ thalassemia may also be compound heterozygotes. For example, a patient may have a $\delta\beta$ thalassemia genotype on one chromosome and a β thalassemia genotype on the other chromosome. These compound heterozygous individuals contribute to the clinical heterogeneity of the thalassemia syndromes. Compound heterozygous $\delta\beta$ thalassemia and β thalassemia produces a clinical course similar to β thalassemia intermedia; however, compound heterozygous hemoglobin Lepore and β thalassemia produces a clinical course similar to β thalassemia major.

Combination Disorders

Although thalassemia has been described in association with a large number of hemoglobin structural variants, the following discussion considers only the interactions with the more common hemoglobin variants (i.e., β thalassemia with hemoglobin S, hemoglobin C, and hemoglobin E, and α thalassemia with hemoglobin S)

β Thalassemia With Hemoglobin S This condition was first recognized in individuals who had inherited a single hemoglobin S gene and demonstrated about 65% hemoglobin S and 35% hemoglobin A, which is the reverse of the proportions found in patients with sickle cell trait. This condition is the result of the inheritance of a hemoglobin S gene from one parent and a β -thalassemia gene from the other. β Thalassemia with hemoglobin S (also called the β -thalassemia sickle cell syndrome) has been widely seen in Africa, the Mediterranean area, the Middle East, and the West Indies. There is great variety in the clinical severity of this syndrome, depending mostly on the type of β -thalassemia gene inherited. If the β -thalassemia gene is the β^0 type, no hemoglobin A is produced, and the clinical condition is similar to classic sickle cell anemia, characterized by severe anemia presenting in early childhood and recurrent sickling crises. If the β -thalassemia gene is the β^+ type, some hemoglobin A is produced, and these patients have less severe sickling crises than those in the β^0 group. The severity of clinical disease reflects the degree of decreased β globin produced by the β^+ mutation.

α Thalassemia With Sickle Cell Anemia The occurrence of α thalassemia in conjunction with sickle cell anemia has a positive influence on the clinical expression of the disease. Patients with such a genetic background have an increased percentage of hemoglobin F, which is thought to result in a decreased severity of the sickling process. Of interest is the fact that the amount of hemoglobin F present is roughly proportional to the number of α -globin genes affected. Patients with the α^0 thalassemia trait have an average of 16% hemoglobin F, and those with the α^+ thalassemia trait have an average of 8% hemoglobin F.¹⁹

β Thalassemia With Hemoglobin C The β thalassemia with hemoglobin C syndrome demonstrates great variability in clinical and hematologic manifestations, which is directly related to the type of β -thalassemia gene that interacts with the hemoglobin C gene; however, the great majority of patients with this syndrome are West Africans and African Americans. In this racial group, the β^+ thalassemia gene is common, and compound heterozygosity for β thalassemia and hemoglobin C is characterized by a mild degree of usually asymptomatic anemia, in which the clinical and hematologic findings are very similar to those found in heterozygous β thalassemia.

β Thalassemia With Hemoglobin E Compound heterozygosity for β thalassemia and hemoglobin E is unusual because it results in a clinical disorder that is much more severe than homozygous hemoglobin E disease. Patients with this syndrome are distributed widely throughout Southeast Asia. The condition follows a clinical course very similar to that of heterozygous β thalassemia, with a very severe anemia occurring

in early childhood and the development of the characteristic features of thalassemia major if the patient is not started on a regular blood transfusion program. In contrast, homozygous hemoglobin E results in microcytosis without significant clinical implication.

Laboratory Diagnosis

The hallmark of thalassemia is the finding of a microcytic, hypochromic anemia. Although more sophisticated laboratory procedures are needed to define exactly the type of thalassemia, the original diagnosis of thalassemia can be made or strongly suspected based on the results of routine hematology procedures.

Routine Hematology Procedures

Automated Blood Cell Analyzer

Automated blood cell analyzers provide hemoglobin levels, hematocrit, and red blood cell indices (see Chapter 32). The red blood cell indices are invaluable to clinicians and laboratory personnel who are sorting through the differential diagnosis of microcytic, hypochromic anemia. Thalassemia minor characterized by a decrease in hemoglobin level, hematocrit, mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH) in conjunction with a normal-to-increased red blood cell (RBC) count, a normal to mildly decreased mean corpuscular hemoglobin concentration (MCHC), and a normal red cell volume distribution width (RDW). In contrast, the RDW is increased in thalassemia major, thalassemia intermedia, and hemoglobin H disease. The decrease in MCV is usually striking and disproportionate to the decrease in hemoglobin and hematocrit. This fact, in conjunction with the relatively high RBC count and the normal RDW, offers a useful discrimination index between heterozygous α or β thalassemia and iron deficiency.²⁰ In iron deficiency, the RDW is increased and the decrease in MCV is less striking and observed only when the anemia is more severe. In heterozygous β thalassemia, the MCH is usually below 22 pg and the MCV below 70 fL, whereas the hemoglobin level is in the 9 to 11 g/dL range.

In heterozygous thalassemia, there is often a relative erythrocytosis. That is, the RBC count is high relative to the hemoglobin level. A general rule is that, in nonthalassemic individuals, the hemoglobin is three times the RBC count. Therefore, a patient with a RBC count of 4 million will have a hemoglobin level of approximately 12 g/dL. However, thalassemic patients have a relative erythrocytosis, so the hemoglobin level will be much less than three times the RBC count. For example, an individual with heterozygous β thalassemia may have an RBC count of 4 million and a hemoglobin level of 9 g/dL. Demonstrating a relative erythrocytosis in an individual with a low MCV should alert the interpreter of a possible thalassemia, and further testing is indicated.

Peripheral Blood Smear Examination

The careful examination of a well-prepared peripheral smear is essential to the diagnosis of thalassemia.

Wright's Stain In homozygous β and compound heterozygous non- α thalassemia, the peripheral smear demonstrates extreme anisocytosis and poikilocytosis with bizarre shapes, target cells, ovalocytes, and large numbers of nucleated red cells (see Fig. 12-4). There is marked hypochromia and microcytosis. In heterozygous β thalassemia, the cells are hypochromic and microcytic with a mild to moderate degree of anisocytosis and poikilocytosis. Target cells are frequent, and basophilic stippling is often seen (see Fig. 12-7). The peripheral smear of a patient with the sickle cell thalassemia syndrome can be differentiated from that of a patient with pure sickle cell anemia by the presence of hypochromia, microcytosis, numerous target cells, and only an occasional sickle cell.

In hemoglobin H disease, the peripheral smear demonstrates hypochromia with microcytosis, target cells, and mild to moderate anisopoikilocytosis. Patients with heterozygous α^0 thalassemia usually demonstrate a mild hypochromia and microcytosis, whereas those with heterozygous α^+ thalassemia usually have a perfectly normal peripheral smear.

Supravital Stains The reticulocyte count is usually elevated up to 10% in hemoglobin H disease and up to 5% in homozygous β thalassemia but is disproportionately low in relation to the degree of anemia in the latter condition.

In hemoglobin H disease, incubation of the red cells with brilliant cresyl blue stain causes in vitro precipitation of hemoglobin H owing to the redox action of the dye. This results in a characteristic appearance of the majority of the red cells, which display multiple discrete inclusions, the appearance of which has often been compared with that of golf balls (see Fig. 12-9). Occasionally, and after extensive searching, such cells containing hemoglobin H inclusions can be found in the α^0 thalassemia carrier.

In patients with homozygous β thalassemia or hemoglobin H disease who have undergone splenectomy, incubation of the blood with methyl violet stain can demonstrate Heinz body-like inclusions, which represent in vivo precipitation of the abnormal hemoglobin.

Acid Elution Stain The acid elution technique (see Chapter 31), originally described by Kleihauer and Betke, is based on the fact that at an acid pH of about 3.3, hemoglobin A is eluted from an air-dried, alcohol-fixed blood smear, whereas hemoglobin F is resistant to elution. After such treatment and subsequent staining with eosin or erythrosin, normal adult red cells appear as very faint ghosts. Red cells containing hemoglobin F demonstrate a variable amount of stain, depending on the amount of hemoglobin F present. A controlled preparation containing a mixture of adult and cord cells must also be stained and examined in parallel to check the quality of the technique, as this technique is very sensitive to many variables. Flow cytometry can also be used to assess hemoglobin F distribution (see later) and may suffer less from the confounding variables of the Kleihauer-Betke test.

CRITICAL THINKING QUESTION

- 12-4 How can the automated hematology results, such as the RBC count and RBC indices, be used to aid the diagnosis of thalassemia?

This stain is very useful in demonstrating the distribution of hemoglobin F and can be used to differentiate between pancellular and heterocellular HPFH. It is also useful in differentiating heterozygous $\delta\beta$ thalassemia from heterozygous pancellular HPFH, because the former usually has a heterocellular distribution of hemoglobin F.

Osmotic Fragility

The red cells of patients with homozygous or heterozygous β thalassemia, hemoglobin H disease, and α^0 thalassemia trait have a decreased osmotic fragility. This fact is not very useful for diagnostic purposes in a specific patient, but it is the basis of a simple, inexpensive method of screening for the thalassemia carrier state in large populations.

Flow Cytometry

Flow cytometry of red blood cells using fluorescently labeled antihemoglobin F antibodies can be used to determine whether the distribution of hemoglobin F is pancellular or heterocellular. Red blood cells are permeabilized and then incubated with antihemoglobin F before being loaded onto the flow cytometer. As red blood cells pass through the laser in single file, they are simultaneously counted and assessed for the presence of hemoglobin F. The end result is either one peak, representing a single population of red blood cells containing hemoglobin F, or two peaks, representing separate populations with and without hemoglobin F.

Flow cytometry is useful in the same situations where Kleihauer-Betke acid elution is useful. Individuals with hemoglobin F in the 20% range may have either heterozygous HPFH or heterozygous $\delta\beta$ thalassemia, and the flow cytometry method can identify the pancellular and heterocellular distribution of hemoglobin F, respectively²¹ (Fig. 12-12).

Hemoglobin Electrophoresis

Hemoglobin electrophoresis has played an important role in the diagnosis of thalassemia by allowing the detection of increased levels of hemoglobin A₂ and hemoglobin F as well as the presence of abnormal hemoglobins. Hemoglobin H, hemoglobin Bart's, hemoglobin Lepore, hemoglobin Constant Spring, or other structurally abnormal hemoglobins can be found in association with thalassemia (hemoglobin S, hemoglobin C, hemoglobin E). Table 12-4 contains a summary of the different patterns of the hemoglobins present in the non- α thalassemia syndromes.

Routine hemoglobin electrophoresis to confirm the diagnosis of thalassemia is performed at an alkaline pH around 8.4 on cellulose acetate, starch, or agarose gels. At alkaline

pH, the hemoglobins migrate from the most cathodal to the most anodal in the following order: first hemoglobin Constant Spring, then hemoglobins A₂, C, and E migrate in the same band; next hemoglobins S and Lepore, again in the same band; then hemoglobin F, followed by hemoglobin A, hemoglobin Portland, hemoglobin Bart's, and finally, hemoglobin H. The different patterns of migration of these hemoglobins are illustrated in Figure 12-8. An example of alkaline electrophoresis on agarose gel with some commonly encountered entities is shown in Figure 12-13. Cellulose acetate or starch gel electrophoresis can be performed at low to neutral pH to detect hemoglobin H and hemoglobin Bart's easily, as they migrate anodally (i.e., in the direction opposite to the other hemoglobins) at this pH. These procedures are useful as a second method of confirmation since primary identification of hemoglobins are performed by high performance liquid chromatography or capillary electrophoresis.

Cellulose Acetate

Cellulose acetate electrophoresis has replaced starch gel electrophoresis in most laboratories, owing to its simple, rapid method. It uses a smaller sample than starch gel electrophoresis, and minor components such as hemoglobin Constant Spring and small amounts of hemoglobin A₂ may be overlooked. Small amounts of hemoglobin A in the presence of mostly hemoglobin F also can be difficult to detect.

Other Gels

Starch gel electrophoresis is a little more cumbersome and time consuming. The results of the starch gel electrophoresis are similar to those of the cellulose acetate procedure. Electrophoresis with starch gel is better at defining the presence of hemoglobin Constant Spring and should always be used if such a variant is suspected.

Citrate agar gel electrophoresis, which is performed at an acid pH between 5.9 and 6.2, is helpful in identifying hemoglobin variants that can be inherited along with thalassemia.

High Performance Liquid Chromatography

Cation-exchange high performance liquid chromatography (HPLC) is commonly used to identify potential hemoglobin F variants and to quantitate hemoglobin A₂ and hemoglobin F. HPLC separates molecules based on their movement through a mobile phase and a stationary phase. The hemoglobin molecule's corresponding retention time in the mobile phase can be used to elucidate its identity. Some advantages of HPLC include its requirement of a smaller sample size compared with hemoglobin electrophoresis, along with its ability to resolve a

FIGURE 12-12 Flow cytometric analysis to determine hemoglobin F distribution. The x-axis represents the amount of hemoglobin in red cells, and the y-axis represents the amount of red blood cells. A pancellular distribution of hemoglobin F in an individual with HPFH is shown in the diagram at left. A heterocellular distribution of hemoglobin F in an individual with heterozygous ($\delta\beta$)⁰ thalassemia is shown in the diagram at right.

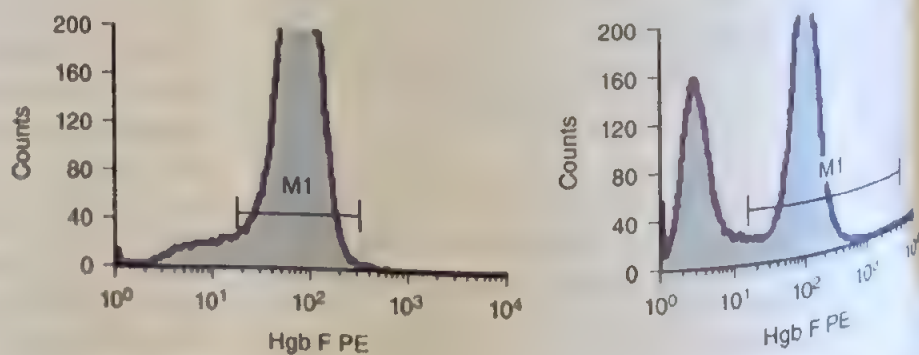


TABLE 12-4 Levels of Hemoglobins A, A₂, and F in the Different Non- α Thalassemias

Type of Thalassemia	HbA (%)	HbA ₂ (%)	HbF (%)
Homozygous β^0 thal	0	2-5	95-98
Homozygous β^+ or compound heterozygous β^+/β^0 thal	5-35	2-5	60-95
Homozygous $\delta\beta$ thal	0	0	100
Homozygous Hb Lepore	0	0	75 (25% Hb Lepore)
Heterozygous β thal	90-95	3.5-7	2-5
Heterozygous $\delta\beta$ thal	80-92	1-2.5	5-20
Heterozygous Hb Lepore	75-85	2	1-6 (7-15% Hb Lepore)
Homozygous HPFH	0	0	100
Heterozygous HPFH (African type)	65-85	1-2.5	15-35
Heterozygous HPFH (Greek type)	75-85	1.5-2.5	15-25
Normal	95-97	2-3	1-2

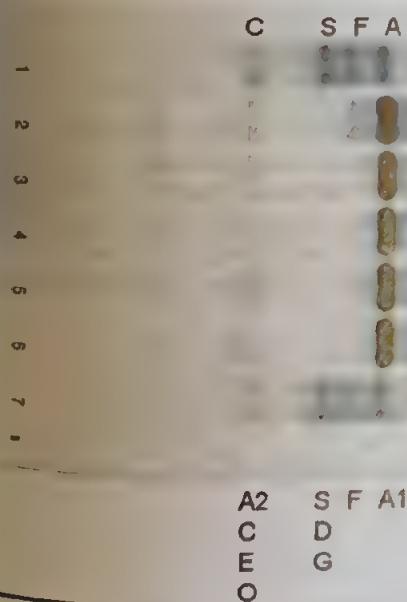


FIGURE 12-13 Electrophoresis at alkaline pH. Lanes 1 and 7 are controls with hemoglobins C, S, F, and A. Lanes 3 and 4 are normal individuals ($A_2 = 2.5\%$). Lane 5 is an individual with β thalassemia minor ($A_2 = 4.7\%$). Lane 6 is an individual with α thalassemia minor; α thalassemia minor has a pattern with a normal A_2 . Lane 2 is an individual with β thalassemia minor ($A_2 = 5\%$) and "Swiss HPFH" ($F_2 = 7\%$).

larger range of hemoglobins. However, it is more costly to perform than hemoglobin electrophoresis, so it is not commonly used in small laboratories. Hemoglobin electrophoresis provides qualitative analysis of Hbs, but HPLC or capillary electrophoresis allows quantitation of the hemoglobin fractions.

Hemoglobin Quantitation

Although an experienced observer can detect an increased level of hemoglobin A₂ or hemoglobin F on cellulose acetate

or starch gel electrophoresis, actual quantitation is necessary to truly establish the diagnosis of thalassemia.

Hemoglobin A₂ Quantitation

The elevation of hemoglobin A₂ is an excellent tool for the detection of a heterozygote carrier of β thalassemia. The level of hemoglobin A₂ ranges between 3.5% and 7% in heterozygous β thalassemia, whereas normal values are always less than 3.5%. A few rare variants of β thalassemia minor with normal or borderline Hb A₂ do exist and can be further investigated with family studies or molecular testing. Also, in an iron-deficient patient with β thalassemia minor, hemoglobin A₂ may be reduced to normal levels.

The percentage of hemoglobin A₂ can be quantified by densitometric scanning of gels after electrophoresis, micro-column chromatography, HPLC, or capillary electrophoresis. HPLC and capillary electrophoresis are commonly used methods for quantitating hemoglobin A₂ because of their superior precision compared with other methodologies. Hemoglobins A, F, S, and C can also be quantitated on some HPLC systems. Limitations of HPLC include the coelution of some hemoglobin variants with hemoglobin A₂, which prevents accurate quantitation, and overestimation of A₂ in carriers of hemoglobin S secondary to hemoglobin S adducts.²²

Hemoglobin F Quantitation

The hemoglobin F levels are useful in the definition of the type of thalassemia involved, and a summary of the levels of hemoglobin F corresponding to the different types of thalassemia can be found in Table 12-4. The hemoglobin F level is normally below 2%. Approximately half of the β -thalassemia carriers have a mildly elevated level of hemoglobin F, usually below 5%.

Routine Chemistry

The indirect bilirubin level is elevated in thalassemia major and intermedia, ranging from 1 to 6 mg/dL. It is characteristically more elevated in thalassemia intermedia than in thalassemia major.

The assessment of the iron status of the patient by the determination of the serum iron level, total iron-binding capacity (TIBC), and serum ferritin level is useful in the differentiation of a thalassemia carrier from a patient with iron-deficiency anemia, as well as in the assessment of the iron load in a patient with thalassemia major or intermedia. The serum iron and serum ferritin levels are low and the TIBC increased in patients with iron deficiency. These values are normal in patients with thalassemia minor unless they have concurrent iron deficiency. Patients with thalassemia major who have been transfused have increased levels of serum iron that approach 100% saturation of the TIBC. The serum ferritin level is elevated and indicates the amount of iron deposited in the tissues.

Differential Diagnosis of Microcytic, Hypochromic Anemia

The differential diagnosis of microcytic, hypochromic anemia includes iron deficiency, α thalassemia, β thalassemia, anemia of inflammation, hemoglobin E disease, sideroblastic anemia, and lead poisoning. Evaluation of the clinical history, hemoglobin level, and red cell indices (in particular, MCV and MCH), and examination of the peripheral smear usually narrow the diagnosis. The sometimes-difficult differentiation of the thalassemia carrier from the iron-deficiency state can be achieved by evaluating the serum iron and ferritin levels and the TIBC. A markedly elevated free erythrocyte protoporphyrin (FEP) identifies a child with lead poisoning. Hemoglobin pattern analysis usually allows differentiation between a β -thalassemia carrier, an α -thalassemia carrier, or the presence of hemoglobin E. The differentiation between these diseases is summarized in Table 12-5.

CRITICAL THINKING QUESTIONS

12-5 Why is it important to differentiate thalassemia minor from iron deficiency anemia?

Treatment

Blood Transfusion

Blood transfusion remains the major form of therapy for symptomatic thalassemia patients. Regular blood transfusions suppress ineffective erythropoiesis and subsequent hemolysis,

lessening patient symptoms associated with each.²³ The aim of regular transfusions is to maintain hemoglobin levels above 10 g/dL.

Due to the vast heterogeneity in clinical presentation of the thalassemias, clinicians have transitioned from classifying thalassemias based on genetic findings to a treatment-based classification. Thalassemia patients are commonly categorized clinically as having transfusion-dependent thalassemia (TDT) or nontransfusion-dependent thalassemia (NTDT). TDT patients require lifelong transfusion to maintain quality of life, whereas NTDT patients may still require transfusion but only in times of crisis such as infection or acute blood loss. Patients with severe forms of thalassemia, such as thalassemia major, fall under the TDT category, whereas patients with less severe forms, such as Hemoglobin H Disease or thalassemia intermedia, typically fall under the NTDT category. However, it is important to note that these categories are fluid, and a patient may move from being TDT to NTDT and vice versa based on many factors.

Three main concerns need to be addressed regarding patients with thalassemia major who are on a regular blood transfusion program:

1. The development of iron overload
2. The development of alloimmunization
3. The risk of transfusion-transmitted diseases

The greatest complication in thalassemia patients receiving regular transfusions is iron overload. Iron overload may lead to organ toxicity and failure, particularly of the heart and liver. Because the body has no way of excreting it, iron chelation therapy is used to rid the body of excess iron. Oral and intravenous drugs used for treatment include deferoxamine, deferiprone, and deferasirox.

Iron overload can also be reduced by increasing the length of survival of the transfused red cells, which requires selection of younger red cells (also called neocytes). Young red cells and reticulocytes have a lower specific gravity than old red cells, and by using a differential centrifugation technique, the blood unit can be separated so that the upper layer of cells is collected. These red cells will have a longer life expectancy and can decrease the transfusion requirement of a patient by lengthening the interval of the blood transfusion schedule.

TABLE 12-5 Differential Diagnosis of Microcytic Hypochromic Anemia

Disorder	RDW	Serum Iron	TIBC	Serum Ferritin	FEP	A ₂ Level
Iron deficiency	↑	↓	↑	↓	↑	nl
α Thalassemia	nl	nl	nl	nl	nl	nl
β Thalassemia	nl	nl	nl	nl	nl	↑
Hemoglobin E disease	nl	nl	nl	nl	nl	nl
Anemia of inflammation	nl	↓	↓	↑	↑	nl
Sideroblastic anemia	↑	↑	nl	↑	↓	nl
Lead poisoning	nl	Nl	nl	nl	↑	nl

RDW = red cell distribution width; TIBC = total iron-binding capacity; FEP = free erythrocyte protoporphyrin; nl = normal.

Alloimmunization is a recurrent problem of all chronically transfused patients. These patients often develop antibodies to both white blood cell and red blood cell antigens. Antibodies to white cell antigens cause febrile nonhemolytic transfusion reactions that are uncomfortable. These reactions can be avoided by routinely transfusing leukocyte-reduced red cells. Alloimmunization to red cell antigens is a more serious problem, because this can cause acute or delayed hemolytic transfusion reactions and may seriously affect the availability of compatible blood. It is recommended that a complete phenotype of the patient's red cells be obtained before embarking on a regular transfusion program.

Transfusion-transmitted diseases are a common complication in multitransfused patients. In the past, patients with thalassemia major often developed hepatitis. This risk has been significantly decreased in many areas of the world with the introduction of testing for hepatitis C. Some patients may develop a chronic form of hepatitis that, in conjunction with the toxicity of iron overload, may result in cirrhosis of the liver.

Other Treatments

Splenectomy

While less common than it once was, splenectomy may also be used in conjunction with, or as an alternative to, transfusion therapy. It may be especially necessary for patients experiencing massive splenomegaly, as in beta thalassemia major. Removal of the spleen allows for a longer RBC lifespan; however, serious complications may accompany a splenectomy. Such complications include increased risk of infection, sepsis, and thrombotic episodes. Splenectomy is not recommended for patients less than 5 years old due to the risk of pneumococcal infection.

Hemoglobin F Stimulation

In the case of β thalassemias, the production of hemoglobin F ($\alpha_2\gamma_2$) can increase the oxygen-carrying capacity of the patient and alleviate the accumulation of free α chains. Hydroxyurea may be used to stimulate the production of hemoglobin F; however, investigation into its efficacy in thalassemia patients is ongoing.²⁴ Other drugs that stimulate gamma chain synthesis may be used to combine with excess alpha chains and decrease alpha chain precipitation and hemolysis.²⁵

Curative Treatment

Blood transfusion and iron chelation is the mainstay of treatment of severe thalassemia. Rigorous adherence to the transfusion and chelation program can allow individuals to survive into the fifth decade; however, many are unable to comply with the regime necessary to achieve maximal medical therapy.

Patient outcomes associated with hematopoietic stem cell transplantation (HSCT) are dependent on patient age, prior treatment, and specificity of HLA-matching donors. The greatest outcomes have been associated with patients under the age of 14. The disease-free survival rates with transplantation of HLA-sibling-matched donor cells exceed 80%.²⁶

The risks of transplantation include acute and chronic graft versus host disease, infections, and graft rejection with the return of thalassemia. Endocrine complications like growth disturbances, infertility, and failure to enter puberty can also

occur after transplantation. Therefore, a careful risk assessment must be made when considering medical management versus transplantation.

New approaches such as gene therapy and gene editing may bring cures with fewer complications. In these instances, the patient's own stem cells and DNA are altered to correct the thalassemia mutation. However, on August 17th, 2022, the U.S. Food and Drug Administration approved Zynteglo, the first cell-based gene therapy for the treatment of adult and pediatric patients with beta thalassemia who require regular red blood cell transfusion. Zynteglo is a one-time gene therapy product administered as a single dose. Each dose is a customized treatment created using the patient's own cells (bone marrow stem cells) that are genetically modified to produce functional beta globin.²⁷

Prevention

The current optimal therapy for thalassemia major relies on intensive use of a sophisticated level of health care. This cannot be achieved in most of the countries where thalassemia major is a serious problem without shunting the major thrust of the health resources in that direction. Another approach to this problem at the health planning level is to decrease the number of births of infants with thalassemia major. This has been successful in certain countries, particularly Cyprus, with the implementation of mass population screening for the detection of heterozygous carriers, genetic counseling, and antenatal diagnosis for couples at risk.

Screening for heterozygous individuals may include an automated blood count with RBC indices, hemoglobin electrophoresis, and estimation of A_2 and F levels. Cut-off values of $MCV < 78$ fL and MCH less than 27 pg have been used to identify candidates for further study. Hemoglobin electrophoresis and estimation of A_2 and F can then be performed to narrow the possibilities, and molecular studies are useful to secure the diagnosis. Developing regions of the world may not have access to sophisticated laboratories, so new simple methods to augment detection are being sought. A single-tube osmotic fragility test may be useful as a screening tool for thalassemia in these countries.²⁸ Other inexpensive alternatives for the detection and quantitation of hemoglobins are being explored.

To ensure understanding of test results and its consequences, proper counseling should be provided to patients who have thalassemia trait. For example, couples who each carry a β -globin gene mutation are at risk of having a child with severe β thalassemia. Perhaps more importantly, women who carry an α^0 thalassemia (—) haplotype are at risk of having a pregnancy complicated by hemoglobin Bart's hydrops fetalis and the associated preeclampsia.

Prenatal diagnosis, using DNA hybridization techniques on sampling from chorionic villi, enables physicians to make a diagnosis during the first trimester of pregnancy. Heterogeneity of thalassemia makes molecular diagnosis difficult; therefore, it is essential to know the ethnicity of mother and father. In this way, the molecular testing can be tailored according to the mutations that are most prevalent in the region of interest.

SUMMARY CHART

- Thalassemia syndromes are one of the most common genetic disorders in the world and are caused by reduced or absent production of globin chains.
- Two main types of thalassemia exist: alpha (α) thalassemia and beta (β) thalassemia.
- Most of the β thalassemias are the result of various genetic mutations. There are two main types of β thalassemia mutations: β^0 thalassemia producing no β globin and β^+ thalassemia producing reduced β globin. In contrast, the majority of α thalassemias result from gene deletions.
- β thalassemia can be divided into three clinical categories: a severe homozygous form with transfusion dependence, β thalassemia major; an intermediate homozygous form, β thalassemia intermedia; and a mild heterozygous form with few symptoms, β thalassemia minor. α Thalassemia can be divided into four clinical categories depending on the severity of the disease: hemoglobin Bart's (hydrops fetalis syndrome), which is lethal; hemoglobin H disease; α thalassemia minor; and silent carrier of α thalassemia.
- Both ineffective erythropoiesis and hemolysis occur in thalassemia; however, ineffective erythropoiesis predominates in severe β thalassemia (β thalassemia major and intermedia) and hemolysis predominates in severe α thalassemia (hemoglobin H disease).
- Hereditary persistence of fetal hemoglobin (HPFH) comprises a group of conditions characterized by the persistence of fetal hemoglobin synthesis into adult life without producing significant hematologic abnormalities.
- The thalassemias are generally characterized by a decrease in hemoglobin level, hematocrit, mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH), in conjunction with a normal to increased red cell count. Peripheral blood smear shows microcytic, hypochromic RBCs, ovalocytes, codocytes, and basophilic stippling. In severe forms, anisocytosis and nucleated red blood cells may also be present.
- Hemoglobin electrophoresis, HPLC, and/or capillary electrophoresis aids in the diagnosis of thalassemia by detecting increased levels of hemoglobin A₂ and hemoglobin F, as well as other abnormal hemoglobins. HPLC and capillary electrophoresis can precisely quantitate these hemoglobins.
- It is imperative to differentiate thalassemia minor and iron deficiency anemia, as both have similar laboratory findings, but are treated differently. A relative erythrocytosis and normal RDW in thalassemia in contrast to an increased RDW and abnormal iron studies in iron deficient anemia can help to differentiate the two disorders.
- Transfusion therapy is the most common treatment for thalassemia major. However, iron overload is a complication that must be controlled using iron chelators. Alloimmunization and transfusion-transmitted disease may also occur with lifelong transfusion therapy. The only current curative treatment for thalassemia is a hematopoietic stem cell transplant although gene editing and gene therapy show promise as future curative options.

CASE STUDY 12-1

A 19-year-old female patient from Bangladesh presents with acute infection indicated by fever and high WBC count with neutrophilia. She has a known history of intermittent jaundice for the past 5 years. A complete blood count gave the following results: RBC, $4.30 \times 10^6/\mu\text{L}$; Hb, 8.3 g/dL; MCV, 76.5 fL; MCH, 19.4 pg; MCHC, 25.4 g/dL; RDW, 18.9%. The peripheral blood smear showed microcytic, hypochromic erythrocytes with a mild anisocytosis, ovalocytes, dacryocytes, and occasional target cells.

Further evaluation reveals the following findings: serum iron is 149 mcg/dL (normal is 60 to 150 mcg/dL); TIBC, 305 mcg/dL (normal is 260 to 360 mcg/dL); and ferritin level, 276 mcg/dL (normal is 30 to 300 mcg/dL). The free erythrocyte protoporphyrin (FEP) is normal. Cellulose acetate electrophoresis shows a slightly decreased hemoglobin A₂ and electrophoresis at alkaline pH showed a fast-moving hemoglobin that appeared as a band anodal to Hb A. Upon

incubation with brilliant cresyl blue, peripheral blood smear showed many "golf ball-like" cells with ragged inclusions.

QUESTIONS

1. Which of the laboratory findings are abnormal and what do the abnormal results indicate?
2. What is the differential diagnosis for these findings, and which can be ruled out using laboratory results?
3. What is the most likely diagnosis and which laboratory result(s) confirm it?
4. What is the significance of the patient's diagnosis?

ANSWERS

1. The hemoglobin and RBC indices indicate a microcytic, hypochromic anemia. The increased RDW indicates slight variation in the size of the RBCs. Poikilocytoses are present on the peripheral blood smear and iron studies are all normal. There is an abnormal

CASE STUDY 12-1—cont'd

hemoglobin present on the gel electrophoresis and abnormal inclusions in the RBCs based on the supravital stain results.

2. Differential diagnosis of microcytic, hypochromic anemia includes alpha and beta thalassemia, iron deficiency anemia, hemoglobin E disease, anemia of inflammation, sideroblastic anemia, and lead poisoning. The RBC count is very helpful for identifying the etiology for this patient's microcytic, hypochromic anemia. In iron deficiency, anemia of inflammation, and sideroblastic anemia, the RBC count is decreased proportionally to the decrease in hemoglobin. Thalassemias have a relative erythrocytosis in which the RBC count is greater than expected in proportion to the hemoglobin value. ($\text{RBC count} \times 3 = \text{expected hemoglobin level in g/dL}$) In anemia of inflammation, the MCV is usually only mildly decreased, and the peripheral blood smear is otherwise normal. The iron studies also help to rule out ACD. The MCV is significantly decreased in well-developed iron deficiency and in thalassemia. The RDW is increased in this patient, which would typically point to iron deficiency anemia rather than a

thalassemia, since a patient with iron deficiency will likely have an elevated RDW. However, normal iron studies rule out IDA. They also rule out sideroblastic anemia, which would show and increase in iron and serum ferritin with a decreased FEP. Lead poisoning would show an increased FEP. The differential diagnosis is limited to alpha or beta thalassemia.

3. The presence of Hb H (the fast-moving hemoglobin anodal to Hb A) on alkaline electrophoresis and the presence of Hb H inclusions on the supravital stain confirm hemoglobin H disease.
4. Milder forms of hemoglobin H disease present episodically during periods of infection, pregnancy, and iron or folic acid deficiency. Diagnosis is critical to the management and prevention of intermittent episodes and for genetic counseling. As a carrier of three nonfunctional alpha genes, female patients with hemoglobin H disease may experience complications during pregnancy and are at an increased risk of bearing a child with hemoglobin Bart's hydrops fetalis if partnered with a carrier of at least one alpha thalassemia gene.

CASE STUDY 12-2

A 22-year-old African American male visits the clinic for a routine, annual physical. He reports to be feeling healthy and well. A complete blood count is performed, and the RBC indices are as follows: RBC, $5.16 \times 10^6/\mu\text{L}$; hemoglobin, 11.4 g/dL; MCV, 71.8 fL; MCH, 22.0 pg; MCHC, 30.7 g/dL; RDW, 14.1%. A peripheral blood smear shows a microcytic, hypochromic red blood cell picture with occasional target cells and basophilic stippling.

Iron studies and an FEP were ordered and are all normal. Hemoglobin electrophoresis was performed and shows 92% hemoglobin A, 5% hemoglobin A₂, and 3% hemoglobin F.

QUESTIONS

1. Which of the laboratory findings are abnormal, and what do the abnormal results indicate?
2. What is the differential diagnosis for these findings, and which can be ruled out using laboratory results?
3. What is the most likely diagnosis, and which laboratory result(s) confirm it?
4. What is the significance of the patient's diagnosis?

ANSWERS

1. The hemoglobin and RBC indices indicate a mildly microcytic, hypochromic anemia. Poikilocytosis and

basophilic stippling are present on the peripheral blood smear, and iron studies are all normal. The hemoglobin electrophoresis results are abnormal, showing a decreased amount of Hb A and slightly increased amounts of Hb A₂ and F.

2. The differential diagnosis is essentially the same as in the first case study. The normal iron studies rule out anemia of inflammation, sideroblastic anemia, and iron deficiency anemia. The normal RDW also helps to rule out IDA. The normal FEP rules out lead poisoning, leaving alpha thalassemia, beta thalassemia, and Hb E disease. The hemoglobin electrophoresis rules out Hb E disease and the alpha thalassemias.
3. Again, the RBC indices are very helpful in supporting a diagnosis of thalassemia, as a relative erythrocytosis is present compared with the low hemoglobin value. The slight decrease in hemoglobin A and slight increase in hemoglobins A₂ and F confirm beta thalassemia minor.
4. It is essential to correctly diagnose both beta and alpha thalassemia minor to avoid unnecessary treatment with iron, which could lead to iron overload. It is also important to identify a carrier of a beta thalassemia gene to provide genetic counseling for the patient before bearing children.

CASE STUDY 12-3

A 1-year-old male from Thailand presents with lethargy, pallor, and splenomegaly. A complete blood count gave the following results: RBC, 4.42 million; hemoglobin, 6.2 g/dL; MCV, 56.0 fL; MCH, 17.0 pg; MCHC, 21.5 g/dL; RDW, 26.0%. A peripheral blood smear shows a microcytic, hypochromic red blood cell picture, marked anisocytosis and poikilocytosis, many nucleated red blood cells, and basophilic stippling. High performance liquid chromatography is the routine hemoglobin testing used in this lab. Upon testing the patient's blood, HPLC showed 0% Hb A, 92% Hb A₂, and 9% Hb F.

Upon repeat testing on cellulose acetate agar at acid pH, a large band appeared in the Hb E position and was quantified to be 89%. Iron studies were also performed and came back normal.

QUESTIONS

1. Which of the laboratory findings are abnormal, and what do the abnormal results indicate?
2. What is the differential diagnosis for these findings, and which can be ruled out using laboratory results?
3. What is the most likely diagnosis, and which laboratory result(s) confirm it?
4. What is the significance of the patient's diagnosis?

ANSWERS

1. The hemoglobin and RBC indices indicate a severe microcytic, hypochromic anemia. The RDW is increased, indicating wide variation in size of the RBCs. The

- peripheral blood picture is striking with marked anisocytosis, poikilocytosis, nRBCs, and basophilic stippling.
2. The differential diagnosis is essentially the same as in the first two case studies. The markedly decreased MCV, severity of anemia, and striking peripheral blood picture rule out anemia of inflammation. The normal iron studies also rule out ACD as well as IDA and sideroblastic anemia. The severity of anemia and striking peripheral blood picture rule out thalassemia minor and indicate a severe form of thalassemia. However, the hemoglobin electrophoresis shows a lack of Hb A, indicating an absence of beta chains, while also showing a large amount of Hb E.
 3. Due to the severity of the anemia, striking RBC picture and large presence of Hb E on electrophoresis, this patient likely has a compound heterozygous inheritance of a β^0 gene and Hb E.
 4. Typically, coinheritance of a thalassemia and hemoglobinopathy results in a mild, asymptomatic, or even positive clinical outcome. Hemoglobin E disease on its own results in a mild clinical course. However, the combination of beta thalassemia and hemoglobin E disease results in a severe anemia, resembling beta thalassemia major. This case is a good example of the heterogeneity of the thalassemia syndromes. It is most common in people of Southeast Asian descent. It is important to identify the precise genetic background of these patients for proper treatment and future genetic counseling.

REVIEW QUESTIONS

1. Which of the following hemoglobin alterations is responsible for the thalassemias syndromes?
 - a. Amino acid substitution
 - b. Abnormal incorporation of iron molecule
 - c. Decreased or absent production of globin chains
 - d. Decreased or absent production of porphyrin rings
2. Which of the following genetic defects is responsible for the majority of α thalassemias?
 - a. Point mutations
 - b. Substitutions
 - c. Insertions
 - d. Deletions
3. What is the clinical expression of a patient compound heterozygous for the β^0 and a severe β^{++} ?
 - a. Thalassemia major
 - b. Thalassemia intermedia
 - c. Thalassemia minor
 - d. Thalassemia minima
4. Which of the following thalassemias is characterized by a variable amount of β_4 hemoglobin, episodic periods of anemia, pallor, and weakness, and characteristic golf ball-like inclusions on a peripheral blood smear stained with brilliant cresyl blue?
 - a. Beta thalassemia major
 - b. Hemoglobin Bart's hydrops fetalis
 - c. Hemoglobin H disease
 - d. Hereditary persistence of fetal hemoglobin
5. Which type of thalassemia has primarily hemoglobin Bart's and shows the following clinical expressions: infants die in utero or soon after birth, severe anemia, marked hepatomegaly and splenomegaly, and ascites?
 - a. Homozygous α^0 thalassemia ($-\alpha/-$)
 - b. Homozygous β^0 thalassemia (β^0/β^0)
 - c. α Thalassemia minor ($\alpha\alpha/-\alpha$)
 - d. Hemoglobin H disease ($-\alpha/-$)

REVIEW QUESTIONS—cont'd

6. Hereditary persistence of fetal hemoglobin (HPFH) exhibits which characteristics?
 - a. Persistence of Hb A₁
 - b. Deletion of the δ - and β -globin genes
 - c. Deletion of α -globin genes
 - d. Always a pancellular distribution of hemoglobin F
7. Which of the following peripheral blood findings is characteristically seen in the thalassemias?
 - a. Microcytic, hypochromic
 - b. Normocytic, normochromic
 - c. Normocytic, spherocytic
 - d. Macrocytic, normochromic
8. Which of the following is a common peripheral blood finding in β thalassemia major?
 - a. Target cells
 - b. Schistocytes
 - c. Macrocytes
 - d. Sick cells
9. Which of the following hemoglobin values is indicative of heterozygous β thalassemia?
 - a. Hemoglobin A level of 65% to 85%
 - b. Hemoglobin A₂ level of 3.5% to 7%
 - c. Hemoglobin A₂ level less than 3.5%
 - d. Hemoglobin F level less than 2%
10. Which of the following laboratory techniques has replaced densitometric scanning of gels after electrophoresis in most high-resource laboratories to quantify hemoglobin A₂?
 - a. Microcolumn chromatography
 - b. High performance liquid chromatography
 - c. Enzyme-linked immune assay
 - d. Osmotic fragility
11. Which of the following sets of laboratory results differentiates heterozygous α or β thalassemia from iron deficiency anemia?
 - a. Heterozygous thalassemia: decreased RDW, with increased MCH and MCV and Hb in the 10 to 14 g/dL range; iron deficiency: increased RDW, MCH, and MCV
 - b. Heterozygous thalassemia: normal RDW, with decreased MCH and MCV and Hb in the 9 to 11 g/dL range; iron deficiency: increased RDW, with decreased MCV and MCH only in severe anemia
 - c. Heterozygous thalassemia: increased RDW, with decreased MCH and MCV and Hb in the 5 to 9 g/dL range; iron deficiency: normal RDW, with normal MCV and MCH
 - d. Heterozygous thalassemia: normal RDW, MCH, and MCV; iron deficiency: RDW, MCH, and MCV all increased
12. What is the most common complication of chronic blood transfusion used to treat thalassemia, and how is it controlled?
 - a. Hyperviscosity of blood; blood thinners
 - b. Iron overload; iron chelators
 - c. Graft versus host disease; HLA matched donor
 - d. Allergic reaction; antihistamine

See answers at the back of this book.

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Rare Normocytic Normochromic Anemias

Aplastic Anemia and Related Disorders and Paroxysmal Nocturnal Hemoglobinuria

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CHAPTER OUTLINE

Introduction

Aplastic Anemia

Pathogenesis

Etiology

Clinical Findings of Aplastic Anemia

Laboratory Evaluation of Aplastic Anemia

Treatment of Aplastic Anemia

Congenital Aplastic Anemia

Pure Red Cell Aplasia

Acquired Pure Red Cell Aplasia

Congenital Pure Red Cell Aplasia:

Diamond-Blackfan Anemia

Congenital Dyserythropoietic Anemias

Paroxysmal Nocturnal Hemoglobinuria

Pathogenesis

Clinical Findings

Laboratory Evaluation

Treatment

Relationships Among Conditions of Bone Marrow Hypoplasia

Summary Chart

Case Study 13-1

Case Study 13-2

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter the learner should be able to:

13-1 Evaluate aplastic anemia in relation to changes seen in the bone marrow.

13-2 Explain the immunological mechanism that causes aplastic anemia.

13-3 List the common causes of acquired aplastic anemia.

13-4 Analyze the congenital bone marrow aplasia that results in aplastic anemia.

13-5 Assess the clinical findings in aplastic anemia.

13-6 Describe the common laboratory results in aplastic anemia.

13-7 Compare the treatment modalities used in aplastic anemia.

13-8 Contrast pure red blood cell aplasia and CDA with aplastic anemia.

13-9 Examine paroxysmal nocturnal hemoglobinuria (PNH).

13-10 Evaluate laboratory results associated with PNH.

13-11 Name the major genetic mutation(s) associated with aplastic anemia and PNH.

This chapter focuses on rare hematological disorders that are normocytic and normochromic in presentation. Aplastic anemia, pure red cell aplasia, dyskeratosis congenita, and congenital dyserythropoietic anemia are disorders that result from various mechanisms targeting bone marrow depletion. The depletion can be due to decreased cellular production, as in aplastic anemia and pure red cell aplasia, or due to the formation of genetically mutated erythrocytes, as in dyskeratosis congenita and congenital dyserythropoietic anemia. These hypoproliferative anemias present with a low number of reticulocytes in circulation.

In contrast, paroxysmal nocturnal hemoglobinuria (PNH) is a normocytic normochromic hemolytic anemia due to a genetically mutated stem cell.

Aplastic Anemia

Aplastic anemia is a rare life-threatening bone marrow failure disorder characterized by the reduction or depletion of cellular elements in the bone marrow.¹ Pancytopenia, which is reduced production of erythrocytes, leukocytes, and platelets, is observed in most cases. The loss of functional hematopoietic stem cells (HSCs) can occur following a variety of bone marrow insults by drugs, chemicals, irradiation, infections, immune dysfunction, and inherited and acquired genetic mutations. Although the inciting mechanisms vary, all lead to the loss of bone marrow precursor cells or damage to the bone marrow itself.¹

Pathogenesis

The basic defect in aplastic anemia is a reduction or cessation of blood cell production by the bone marrow. Blood cell production is dependent on the growth, differentiation, and self-renewal of a common, pluripotential **hematopoietic stem cell (HSC)**. The hematopoietic stem and progenitor cell is identified by CD34 + surface antigens. The CD34 + population in patients with aplastic anemia is significantly lower than those found in healthy individuals. Under normal conditions, the hematopoietic stem and progenitor cells proliferate and differentiate in response to cytokines and other growth factors produced in the bone marrow microenvironment (see Chapter 1, Hematopoiesis). Patients with aplastic anemia are found to have higher than normal levels of growth factors in the bone marrow. Regardless, hypoproliferative bone marrow develops as a result of decreased self-renewal or cellular destruction. Alternatively, damage to the bone marrow by a variety of influences could lead to bone marrow aplasia (Fig. 13-1). Most studies point to a decrease in HSCs rather than a defective microenvironment as the underlying defect in aplastic anemia. Box 13-1 outlines the pathogenic mechanisms in the development of aplastic anemia.¹

Etiology

Aplastic anemia is a rare diagnosis and typically includes pancytopenia, reticulocytopenia, bone marrow hypocellularity, and reduction of hematopoietic stem and progenitor cells. Clinically, it is useful to divide aplastic anemia into acquired or congenital (hereditary) types. The vast majority of cases are acquired.² Select cases of acquired aplastic anemia have been reported from documentable exposure to chemicals, drugs, irradiation, or infection. However, the majority of cases are idiopathic. Hereditary cases of aplastic anemia are extremely rare, with the most common group designated as Fanconi's anemia.³

Acquired Idiopathic Aplastic Anemia

Acquired aplastic anemia, also termed idiopathic, is responsible for 70% of all newly diagnosed cases.² The anemia is idiopathic in nature because no clear-cut primary cause of the bone marrow failure can be identified. However, current research supports a theory that most instances of aplastic anemia are caused by a type of immune dysfunction, specifically autoimmunity. Imbalances in the numbers of several types of T lymphocytes have been found in idiopathic cases.² An increased population of oligoclonal **cytotoxic T lymphocytes** and a lack of **regulatory T lymphocytes (T cells)**

BOX 13-1 Pathogenic Mechanisms in Development of Aplastic Anemia

Immune-Mediated Bone Marrow Damage

- Autoimmunity
- Pregnancy
- Infection

Direct Bone Marrow Toxic Effects

- Radiation
- Drugs (i.e., chemotherapy drugs, other drugs)
- Benzene

Congenital Bone Marrow Defects

- Fanconi's anemia
- Dyskeratosis congenita

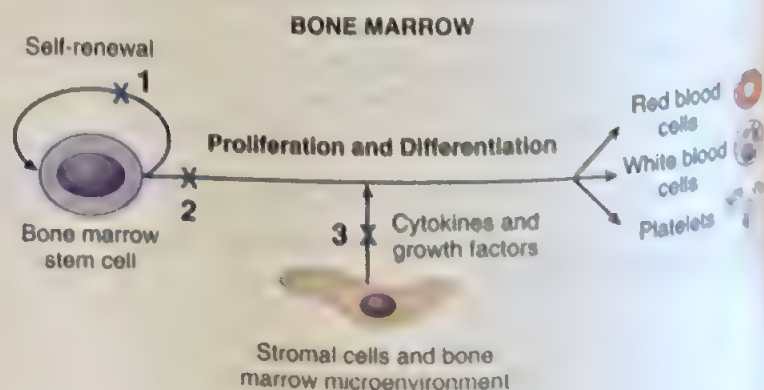
have been observed.² Recent advances suggest that immune-mediated suppression and destruction of hematopoietic stem cells (HSCs) and hematopoietic stem cell progenitors (HSPC) are two explanations for the cause of aplastic anemia.^{4,5}

Populations of activated cytotoxic T lymphocytes attack and destroy HSCs in the marrow. As the population of HSCs decrease, so do the numbers of erythrocytes, granulocytes, and platelets in the peripheral blood.^{5,6} In turn, regulatory T cells, the cells that regulate the development of other T-cell populations that should suppress autoreactive T cells, are lacking.^{6,7} The theory that aplastic anemia is an autoimmune disease is supported by the fact that most aplastic anemia patients respond to immunosuppressive therapy.¹ In fact, immunosuppressive therapy targeting T cells leads to a response in two-thirds of patients with idiopathic aplastic anemia.⁸

Etiology of Environmental Agents

Chemical Agents Some of the chemical agents linked with aplastic anemia include benzene, trinitrotoluene, arsenic, insecticides, and weed killers. Many of these compounds have a benzene ring, which is highly associated with development of aplastic anemia. Benzene is used in the manufacture of plastics, drugs, dyes, and explosives. Because most benzene compounds are volatile, they are easily absorbed by inhalation. The development of bone marrow aplasia varies among those exposed and is dosage-related. Often, bone marrow suppression is reversible after discontinuation of benzene exposure.

FIGURE 13-1 Schematic representation of possible defects in hematopoiesis that may give rise to aplastic anemia. It is postulated that decreased numbers of bone marrow stem cells and/or changes in the bone marrow microenvironment that alter cytokine levels, or both, may cause aplasia to develop. Most evidence points to decreased stem cells caused by the lack of self-replication (1) or direct destruction of stem cells (2), rather than changes in the bone marrow microenvironment (3), as pathogenic mechanisms for development of aplastic anemia.



Drugs A wide variety of drugs have been associated with development of aplastic anemia. The antibiotic chloramphenicol and the anti-inflammatory drug phenylbutazone are probably the best documented examples of drugs associated with aplastic anemia. The toxicity associated with these drugs is usually *not* related to the total dosage of the drug received. It is impossible to identify which patients will react adversely to a drug. Luckily, such idiosyncratic reactions to drugs are relatively rare. Because of the association with development of aplastic anemia, chloramphenicol use has decreased.^{1,8}

A number of other drugs have been associated with development of bone marrow aplasia, but a causal effect is often difficult to verify. The incidence and predictability of bone marrow suppression vary with the type of drug and the individual's ability to metabolize the drug. (Table 13-1; Fig. 13-2). Often, drug-induced bone marrow hypoplasia is fully reversible upon removal of the drug.^{1,7}

Ionizing Radiation It has long been known that ionizing radiation has a destructive effect on the cells of the bone marrow. High doses of radiation lead to complete loss of hematopoietic cells that is irreversible and lethal. Lesser doses lead to reversible anemia, leukopenia, and thrombocytopenia with full recovery of counts in 4 to 6 weeks. Hematopoietic cells are most susceptible to penetrating forms of radiation, such as those found in gamma rays and x-rays. However, chronic ingestion of lower-energy

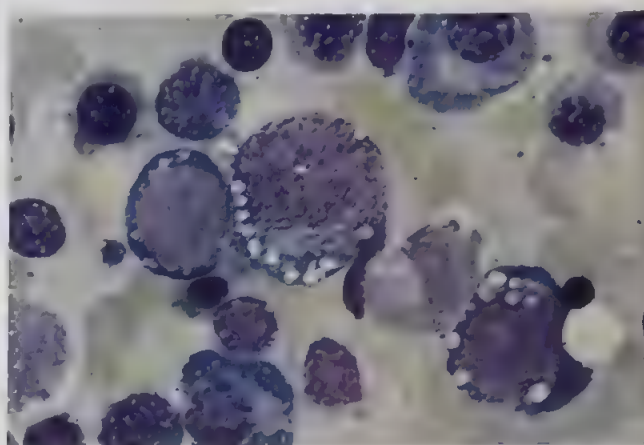


FIGURE 13-2 Vacuolization of bone marrow hematopoietic precursor cells indicating toxicity in a patient being treated with chloramphenicol. (Wright-Giemsa stain, $\times 1,000$ magnification)

radiation sources may also cause deleterious bone marrow effects. Aplastic anemia may occur months to years after radiation exposure, although development of bone marrow dysplasia and acute leukemias is more common.¹

Infection Many infections have suppressive effects on the bone marrow. Acute, self-limited infections may suppress bone marrow activity for 10 to 14 days with only minor effects on the

TABLE 13-1 Agents Associated With Aplastic Anemia

Agents That Regularly Produce Bone Marrow Hypoplasia With Sufficient Doses

- Ionizing radiation
- Benzene and benzene derivatives
- Chemotherapeutic agents (e.g., busulfan, vincristine)

Drugs That Produce Bone Marrow Hypoplasia in an Idiosyncratic Manner

Type of Drug	Relatively Frequent	Rare
Antimicrobials	Chloramphenicol	Streptomycin
	Penicillin, tetracycline	Amphotericin B
		Sulfonamides
Anticonvulsants	Methylphenylethylhydantoin	Methylphenylhydantoin
	Trimethadione	Diphenylhydantoin
		Primidone
Analgesics	Phenylbutazone	Aspirin
		Tapazole
Hypoglycemic agents		Tolbutamide
		Chlorpropamide
Insecticides		Chlorophenothane
		Parathion
Miscellaneous		Colchicine
		Acetazolamide
		Hair dyes

peripheral blood counts. Several viral infections, including hepatitis, Epstein-Barr virus, and cytomegalovirus,^{9,10} have been associated with the development of aplastic anemia. Of these, hepatitis from an uncharacterized hepatitis virus (i.e., non-A, non-B, non-C type) has the strongest association with the development of aplastic anemia. The mechanism whereby viruses cause aplastic anemia is unknown. It has been suggested that the hepatitis non-A, -B, -C virus may induce an autoimmune reaction against bone marrow hematopoietic stem cells.¹¹

Other Causes States of altered immune functions, such as pregnancy,¹² systemic lupus erythematosus, or graft versus host disease post-bone marrow transplant, have been associated with development of aplastic anemia. Other cases of aplastic anemia have been associated with vitamin deficiency and copper deficiency in patients suffering from malnutrition.⁹

Clinical Findings of Aplastic Anemia

The development of aplastic anemia is often an insidious process and typically occurs in the young or elderly.^{7,8,9} There is a bimodal age of peak presentation, with the first occurring in patients aged 15 to 25 years and the second in patients over 60 years old.⁵ In the United States and Europe, there are about 2 cases per million, while in East Asia there are 4 to 6 times more cases.^{2,7,8} In the bone marrow, the gradual decrease in hematopoietic stem and progenitor cells results in a corresponding decrease in erythrocytes, leukocytes, and platelets in the peripheral blood. Most patients present with symptoms of anemia such as fatigue, dyspnea, or exercise intolerance. Physical examination may reveal pallor due to the anemia or evidence of thrombocytopenia, such as petechiae, purpura, or ecchymoses. Signs of infection such as fever, due to the decrease in leukocytes, may also be present. Other physical findings are minimal. A detailed history of drug ingestion, toxic exposure, or infection is essential, as well as a family history of similar hematologic problems.^{7,8}

Laboratory Evaluation of Acquired Aplastic Anemia

Diagnosis of aplastic anemia should include tests to determine the degree of bone marrow dysfunction and assays to rule out other possible causes of the blood cytopenias. Initial testing includes a complete blood count (CBC), reticulocyte count, peripheral smear, and bone marrow examination. Tests to evaluate renal and hepatic function, as well as cultures or serological tests looking for infectious agents, are also recommended. Laboratory evaluation for aplastic anemia is summarized in Table 13-2.^{7,9}

The CBC shows varying degrees of pancytopenia, with a hemoglobin concentration of 7.0 g/dL or lower. The red blood cells are usually normochromic and normocytic with the MCV and MCHC falling within the reference range. The corrected reticulocyte count is low, less than 1%, or an absolute reticulocyte count of less than $25 \times 10^9/L$, indicating a lack of bone marrow activity. The white blood cells, particularly the myeloid and monocytic cells, are decreased while lymphocytes may be normal or decreased in number. The platelet count is also decreased.

Clinical criteria that have been used to define aplastic anemia include marrow of less than 25% normal cellularity and

TABLE 13-2 Laboratory Evaluation for Aplastic Anemia

Test	Purpose
CBC and differential	Establish severity of cytopenias
Peripheral blood examination	Exclude malignancy and other causes of cytopenias
Reticulocyte count	Establish decreased marrow regeneration
Bone marrow examination	Rule out leukemia, other causes of cytopenias (i.e., myelodysplasia, storage disorder, metastatic diseases, granulomas, fibrosis); establish hypoplasia of bone marrow
Biochemical testing	Liver function, renal function
Cultures	Document possible infection
Serological testing	Document infection

at least two peripheral blood cytopenias. Those cytopenias include:

1. absolute neutrophil count (ANC) $<0.5 \times 10^9/L$
2. platelets $<20 \times 10^9/L$ or corrected reticulocyte count $\leq 1\%$ in the presence of a hypocellular bone marrow (absolute count $\leq 20 \times 10^9/L$)

Examination of the blood smear confirms a normochromic, normocytic anemia with no apparent abnormal red cell morphology. Polychromatic cells, basophilic stippling, or nucleated red cells are absent. White cells usually show a relative lymphocytosis of as much as 70% to 90%, reflecting decreased numbers of myeloid and monocytic cells. Immature myeloid cells, such as myelocytes or metamyelocytes, if present, would point to a diagnosis of disorders other than aplastic anemia. Platelets are usually decreased, and it is unusual to find large or abnormal forms.

Because the CBC and peripheral blood smears offer few clues to the cause of the pancytopenia, a bone marrow aspiration and core biopsy must be performed to rule out other disorders. The bone marrow aspiration is often markedly hypocellular (Fig. 13-3). Small numbers of lymphocytes, plasma cells, and rare hematopoietic precursor cells are seen. The biopsy specimen is typically very hypocellular with marked reductions of myeloid, erythroid, and megakaryocytic progenitors with fatty replacement of hematopoietic tissue.¹ Compared with a normocellular bone marrow biopsy specimen (Fig. 13-4A), a markedly hypocellular bone marrow biopsy sample shows only residual stroma and fat (Fig. 13-4B). Scattered lymphocytes and plasma cells (Fig. 13-5) or occasional lymphoid aggregates (Fig. 13-6) may be seen (Fig. 13-7). Using the results of the CBC, reticulocyte count and the severity of the condition is graded as nonsevere, severe, or very severe^{7,9,12} (see Table 13-3).

CRITICAL THINKING QUESTION

13-1 Why would the reticulocyte count in aplastic anemia be low?

See answers to all Critical Thinking Questions at the back of this book.

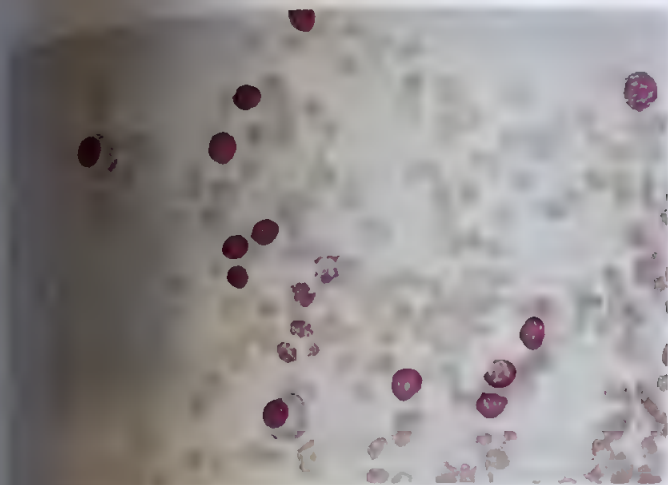


FIGURE 13-3 Hypocellular bone marrow aspirate containing primarily lymphocytes and plasma cells, reflecting bone marrow aplasia. (Wright-Giemsa stain, $\times 500$ magnification)

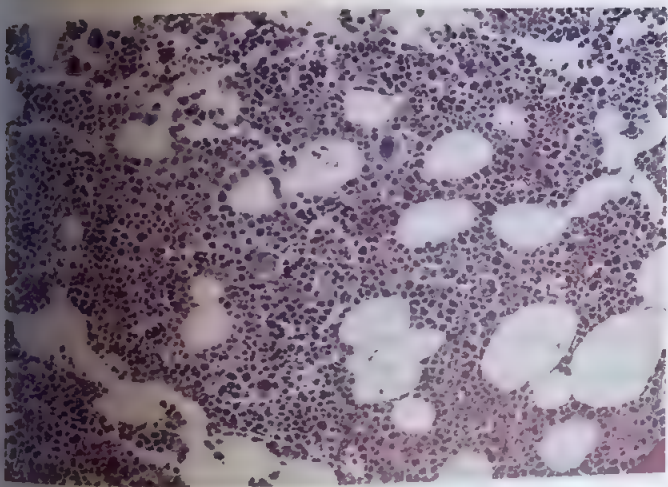


FIGURE 13-4 **A.** Normocellular bone marrow. **B.** Markedly hypocellular bone marrow biopsy specimen from a patient with aplastic anemia. (H & E stain, $\times 500$ magnification)

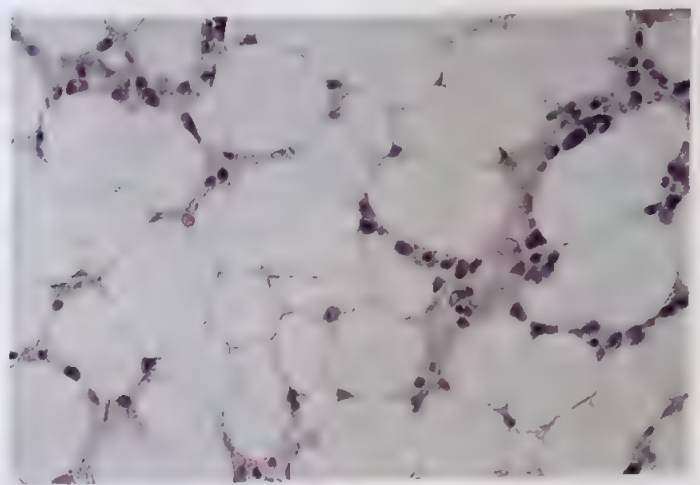


FIGURE 13-5 Residual lymphocytes, plasma cells, and bone marrow stroma with marked decrease in hematopoietic cells in a bone marrow biopsy specimen from a patient with aplastic anemia. (H & E stain, $\times 200$ magnification)

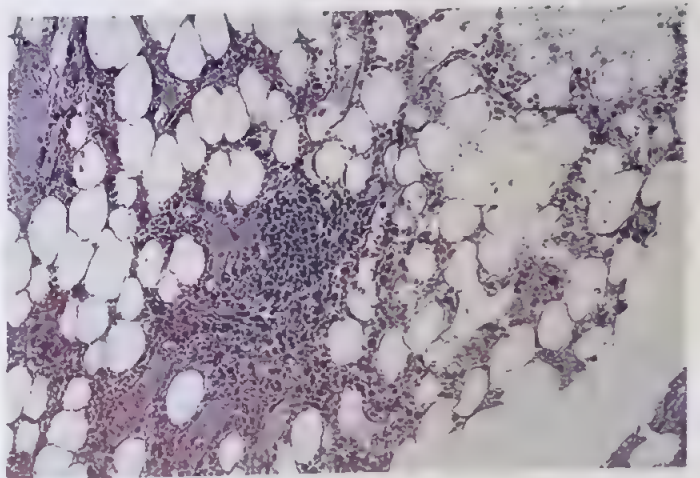


FIGURE 13-6 Lymphoid aggregate seen in a bone marrow biopsy specimen from a patient with aplastic anemia. (H & E stain, $\times 100$ magnification)

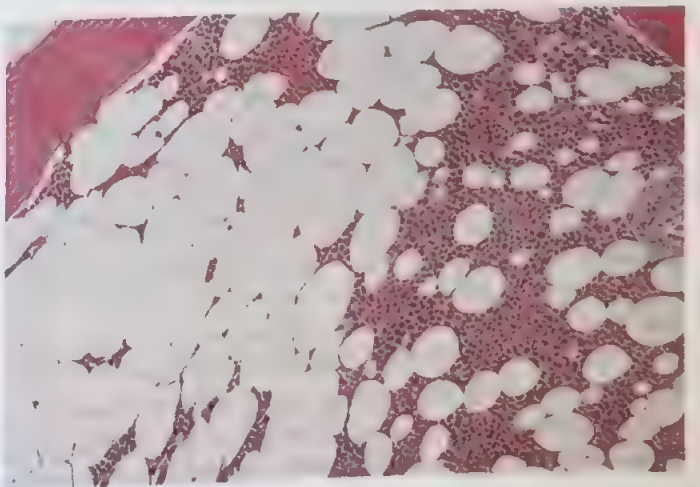


FIGURE 13-7 Focal area of bone marrow hyperplasia adjacent to a hypocellular area in early aplastic anemia. (H & E stain, $\times 100$ magnification)

Treatment of Aplastic Anemia

Untreated AA has an extremely poor prognosis, as the patients undergo progressive decreases in blood counts and subsequent lethal infection or bleeding. Characteristic abnormal CBC values seen in severe aplastic anemia are listed in Box 13-2.

Currently, the treatment of choice for AA in patients younger than 50 years of age is allogeneic bone marrow transplantation. This therapy is optimal if bone marrow from an HLA-matched sibling is used, although HLA-matched unrelated donors may also be considered.^{1,9,13} Long-term survival

TABLE 13-3 Classification of Aplastic Anemia by Severity

Classification	Bone Marrow	Neutrophils	Platelets	Reticulocytes
Moderate AA	<30% cellularity plus ≥ 2 cytopenias	$<1.0 \times 10^9/L$	$<50 \times 10^9/L$	$<1\%$ or $<20 \times 10^9/L$
Severe AA	<25% cellularity plus at least 2 cytopenias	$<0.5 \times 10^9/L$	$<20 \times 10^9/L$	$<1\%$ or $<20 \times 10^9/L$
Very Severe AA	<25% cellularity neutropenia plus one other cytopenia	$<0.2 \times 10^9/L$	$<20 \times 10^9/L$	$<1\%$ or $<20 \times 10^9/L$

BOX 13-2 Characteristic Abnormal CBC Values Seen in Severe Aplastic Anemia

Red Blood Cells

- Hematocrit ≤ 0.20 – 0.25 (L/L) or 20%–25%
- Hemoglobin concentration ≤ 70 g/L
- Absolute reticulocyte count $\leq 25 \leq 10^9/L$
- Corrected reticulocyte count $<1\%$

White Blood Cells

- Total leukocyte count $\leq 1.5 \times 10^9/L$
- Absolute neutrophil count $\leq 0.5 \times 10^9/L$

Platelets

- Platelet count ≤ 20 – $60 \times 10^9/L$

rates of 90% in young children and 80% in adolescents¹ have been reported following bone marrow transplantation, usually with full functional bone marrow recovery. However, for children with severe aplastic anemia who lack an HLA-identical sibling, the standard treatment remains immunosuppressive therapy with horse antithymocyte globulin (ATG) and cyclosporine.¹⁴ The success of transplantation decreases in patients who are over the age of 40, and for those who have had multiple blood transfusions, likely due to autoimmunization. Therefore, bone marrow transplant should be considered early in the course of treatment. Up to 75% survival after 5 years has been seen with this therapy, although relapses can occur.³ If a transfusion is deemed necessary, Rh and Kell phenotype-matched, leuko-reduced blood products are recommended.^{1,9,10}

For all patients who are unable to receive bone marrow transplantation because of older age or lack of a suitable donor, immunosuppressive therapy is recommended. ATG contains antibodies that deplete activated T cells. If a relapse occurs, patients may be treated with cyclosporine alone or a second round of either horse or rabbit ATG.¹³ If a patient treated with immunosuppression needs transfusion, the blood should be irradiated.¹³ A third drug, eltrombopag, a thrombopoietin receptor agonist, is now being used in conjunction with ATG and cyclosporine.¹⁴ Patients receiving eltrombopag showed greater hematologic improvement than those receiving the standard therapy alone.^{6,14}

The prognosis for patients with aplastic anemia has markedly improved since options as stem cell transplantation and immunotherapy have become available. The outcome is still variable and depends primarily on the severity of the anemia at the time of presentation, the age of the patient, supportive care

such as blood product transfusions, and the treatment modality employed.^{1,9,13}

Congenital Aplastic Anemia

Fanconi's Anemia

More than 25% of pediatric patients and 5% to 15% of adults age 40 or younger who present with aplastic anemia have an inherited etiology.² The most common form of congenital bone marrow aplasia is **Fanconi's anemia**.¹⁵ It is a rare, autosomal recessive disorder in which patients have a variety of associated developmental abnormalities, including one or more of the following: skeletal defects, cutaneous hyperpigmentation, renal abnormalities, microcephaly, intellectual disability, and poor growth.¹⁵ Usually, patients develop symptomatic pancytopenia between the ages of 5 to 10 years.¹⁵ The bone marrow may be originally normocellular or hypercellular, but over time hypoplasia develops.

Fanconi's anemia patients display genetic mutations or deletions in multiple genes. At least 22 genes are associated with Fanconi's anemia.¹⁶ In the majority of cases, biallelic mutations involving one or more of the 22 genes that have been determined to participate in DNA repair and genome stability, termed the *FANCA* gene complementary group, is responsible for the disease.¹⁶ Laboratory results are similar to patients with aplastic anemia. The chromosomes of Fanconi's anemia patients are unstable and susceptible to breakage, which is thought to lead to loss of HSCs in the marrow. This may explain why these individuals have an increased susceptibility to development of cancer.¹ Diagnostic tests include an analysis of chromosomal breakage and genetic testing. Untreated patients with FA usually die of infections or hemorrhage secondary to blood cytopenias or development of malignancy.¹⁷

Dyskeratosis Congenita

Another, less common inherited condition associated with aplastic anemia is Dyskeratosis Congenita. These patients present with abnormal skin pigmentation, dystrophic nails, and oral leukoplakia (white lesions that form in the mouth). The majority of patients develop bone marrow abnormalities by middle age, and they have an increased risk of cancer.

Dyskeratosis Congenita patients have short telomeres. A telomere analysis using flow cytometry, fluorescence in situ hybridization, or polymerase chain reaction is done on patients suspected of having Dyskeratosis Congenita. The genetic defect in Dyskeratosis Congenita involves a mutation in one of 11 telomere genes, and the defect affects telomere maintenance and repair affecting the HSC's ability to repair DNA. Laboratory findings include pancytopenia, macrocytic erythrocytes, and an increased level of fetal hemoglobin. The majority

of Dyskeratosis Congenita patients will die of complications associated with bone marrow failure.

Pure Red Cell Aplasia

Pure red cell aplasia is a rare disorder in which the erythroid precursors in the bone marrow are selectively destroyed. In contrast to aplastic anemia, pure red cell aplasia only affects the red blood cell line, while WBCs and platelets remain at normal levels. This produces a normocytic, normochromic anemia with severe reticulocytopenia without leukopenia or thrombocytopenia. This may be an acquired or congenital process (Box 13-3). Pure red cell aplasia is characterized by a severe, chronic, normocytic anemia. Reticulocytes are not only decreased but may be absent in the bone marrow. There is no evidence of hemolysis or hemorrhage. Bone marrow biopsy shows normal cellularity with an absence of erythroid precursors. EPO levels are often markedly increased as the body attempts to compensate for the anemia.¹⁸

Acquired Pure Red Cell Aplasia

Acquired pure red cell aplasia can be either a primary (idiopathic) disorder or secondary to some other disorder, infection, or drug. Primary acquired pure red cell aplasia is considered to be an autoimmune disorder.¹⁸ The most common type of secondary pure red cell aplasia is transient or acute self-limited, and is usually precipitated by a virus or drug.¹⁸ In most cases, the pure red cell aplasia will resolve when the drug is stopped or the infection no longer exists.

Viral illnesses have been associated with development of a transient disappearance of erythroblasts from the bone marrow. Parvovirus B19 infection, the causative agent of erythema infectiosum, or fifth disease has been associated with transient red cell aplasia in children.¹⁹ Most patients are relatively asymptomatic; however, in patients with long-standing hemolytic disease (e.g., sickle cell disease, hereditary spherocytosis), this loss of red cell production causes a sharp decrease in hematocrit known as an aplastic crisis. Parvovirus B19 selectively infects red blood cell precursors, leading to a lack of red cell production. Some erythroblasts may contain viral inclusions (Fig. 13-8). Except for some immunocompromised

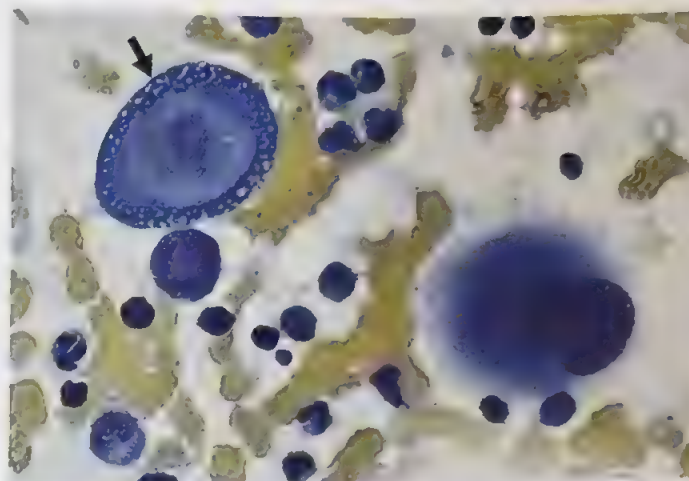


FIGURE 13-8 Erythroid precursors containing parvovirus B19 viral inclusions. (Wright-Giemsa stain, $\times 500$ magnification)

patients, most recover erythropoietic capacity, as demonstrated by the appearance of reticulocytes, within 7 to 10 days.¹⁹

Another frequent cause of acquired secondary pure red cell aplasia is an idiosyncratic reaction to a drug. Several drugs have been implicated, and these are usually the same drugs that give rise to AA, including phenytoin, isoniazid, and azathioprine. Thymoma is also associated with red cell aplasia, especially in adults.^{18,20}

Congenital Pure Red Cell Aplasia: Diamond-Blackfan Anemia

Diamond-Blackfan anemia is a rare inherited form of pure red cell aplasia characterized by congenital bone abnormalities and a chronic macrocytic-normocytic anemia.²¹ It is typically a moderate to severe anemia that usually manifests early in infancy, usually before age 1. Diamond-Blackfan anemia is associated with low reticulocytes and normal numbers of white cells and platelets. Bone marrow examination shows a normocellular bone marrow with erythroid hypoplasia. This anemia is a rare disease with an incidence rate of 1 in 500,000 live births.²² Diamond-Blackfan anemia is a result of a genetic mutation, the most common mutation involving a ribosomal protein on chromosome 11. Of the cases, 60% to 70% have a ribosomal gene mutation present and are inherited as autosomal dominant in 40% to 45% of patients. It is not known why only erythroid precursors are affected by the ribosomal protein mutations.²³

Congenital Dyserythropoietic Anemias

Congenital dyserythropoietic anemias (CDAs) are a rare group of familial disorders characterized by ineffective erythropoiesis. A unique finding in CDAs is the presence of bizarre binuclear and multinuclear erythroblasts in the bone marrow (Fig. 13-9). Three types of CDA have been well described (Table 13-4). All CDAs present clinically with mild to moderate anemia, erythroid hyperplasia with variable degrees of dyserythropoiesis, and indirect hyperbilirubinemia or mild jaundice.^{24,25,26}

CDA type I is a mild to moderate macrocytic anemia with prominent anisocytosis and poikilocytosis. Bone marrow

BOX 13-3 Causes of Pure Red Cell Aplasia

Acquired

- Infections—parvovirus B19
- Aplastic crisis in patients with hemolytic disorders
- Immunocompromised patients
- Malnutrition
- Drugs
- Direct toxicity
- Development of antibodies to red cell precursors or EPO
- Thymoma or lymphoid neoplasms
- Idiopathic

Congenital

- Diamond-Blackfan anemia

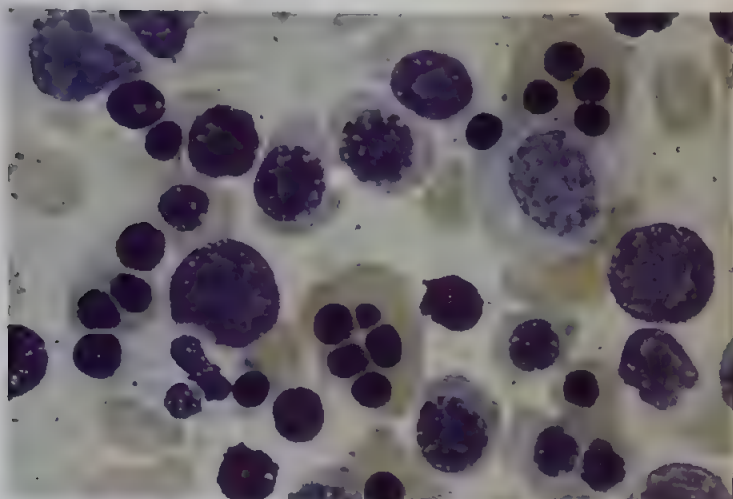


FIGURE 13-9 Multinucleated erythroid precursors in type 2 congenital dyserythropoietic anemia (HEMPAS). (Wright-Giemsa stain, $\times 1,000$ magnification)

erythroblasts show megaloblastic maturation and the presence of a small number (1% to 3%) of marrow erythroblasts that are binucleated or contain chromatin bridges (thin, fiberlike connections between the nuclei) (Fig. 13-10).²⁴

CDA type II is the most frequently seen form of CDA. Patients are found to have a mild to severe normocytic anemia in which 10% to 50% of the erythroblasts are binucleated or multinucleated (see Fig. 13-9).^{24,25,26} CDA type II red cells will lyse in the acidified serum test (described later), giving rise to the alternative name of *HEMPAS* (hereditary erythroblast multinuclearity with a positive acid serum test). Because cells from patients with paroxysmal nocturnal hemoglobinuria also produce a positive acidified serum test, PNH must be ruled out. Unlike PNH, the cells of CDA type II do not lyse in a sugar water test, nor do they have the decreased levels of membrane CD55 and CD59 on flow cytometry. In addition, the *i* antigen is persistently expressed on all type II CDA cells, so they strongly agglutinate with anti-*i* antibody.²⁶



FIGURE 13-10 Binucleated erythroid precursor with chromatin bridge seen in a patient with type I congenital dyserythropoietic anemia (Wright-Giemsa stain $\times 1,000$ magnification)

CDA type III presents as a mild to moderate macrocytic anemia. It differs from type I CDA by having as many as 30% multinucleated bone marrow erythroid cells, some containing as many as 12 nuclei (gigantoblasts).²⁴

Paroxysmal Nocturnal Hemoglobinuria

Paroxysmal nocturnal hemoglobinuria (PNH) is a normocytic normochromic hemolytic anemia, with increased reticulocytes in circulation. PNH cells can also be present in patients

TABLE 13-4 Types of Congenital Dyserythropoietic Anemias

Characteristic	Type I	Type II	Type III
Inheritance pattern	Autosomal recessive	Autosomal recessive	Familial: autosomal dominant Sporadic: variable
Red cells	Macrocytic	Normocytic	Macrocytic
Anemia	Mild to severe	Mild to moderate	Mild to moderate
Light microscopy-marrow	Intranuclear chromatin bridges	Binucleation	Giant erythroblasts
Electron microscopy	"Swiss cheese" nuclei	Peripheral cisternae	No specific findings
Acidified serum lysis	Negative	Positive	Negative
Genetic localization	15q15.-15.3	20q11.2	Familial: 15q22 Sporadic: variable
Postulated biochemical defect	Unknown	Underglycosylation of band 3	Unknown
Associated abnormalities	Skeletal defects, skin hypopigmentation	None	Familial: visual defects monoclonal gammopathy Sporadic: association with lymphoma mental retardation

with aplastic anemia. These cells are particularly sensitive to lysis by complement due to a genetically mutated stem cell. Because of the many mutations and mechanisms involved in these disorders, accurate diagnosis is extremely important for effective treatment to take place.

Pathogenesis

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare, acquired stem cell disorder that results in abnormalities of the red cell membrane.²⁸ This causes the red cells to be highly sensitive to complement-mediated hemolysis. Because this is a stem cell disorder, abnormalities are also present in leukocytes and platelets. PNH is characterized by recurrent episodes of intravascular hemolysis, hemoglobinuria, and venous thrombosis. PNH is also strongly associated with AA (discussed later).^{27,28}

PNH arises from a somatic mutation in the HSC that gives rise to complete or partial deficiencies of cell surface **glycophosphatidylinositol (GPI) anchor proteins (GPIAPs)**. The mutated gene is **phosphatidylinositolglycan A (PIGA)**, which is located on the X chromosome. The deficiency of GPI anchor proteins causes variable deficiencies in at least 150 cell surface proteins (Box 13-4). These surface proteins include two membrane glycoproteins that regulate complement fixation and activation on the cell surface, CD55 (decay-accelerating factor or DAF), and CD59 (membrane inhibitor of reactive lysis or MIRL). CD55 regulates the activity of C3 convertase, and CD59 prevents pore formation by the membrane attack complex (MAC).²⁷ A deficiency of either of these proteins leads to increased complement-mediated hemolysis. White blood cells and platelets can also demonstrate abnormal cell surface GPI anchor protein levels. The amount GPI anchor proteins present may vary, with some cells expressing normal levels and other cells expressing lesser amounts. This has led to description of three different subtypes of PNH cells (Table 13-5). The relative numbers of each PNH cell type correlate with disease severity. A patient may have some or all of these subtypes present, and the numbers of each subtype may shift over the course of the disease.^{28,29}

BOX 13-4 Hematopoietic Cell Surface Proteins Decreased or Absent in Paroxysmal Nocturnal Hemoglobinuria Patients

Complement Regulatory Proteins

- Decay accelerating factor (CD55)
- Homologous restriction factor
- Membrane inhibitor of reactive lysis (CD59)

Proteins Associated With Immune Function

- Lymphocyte function antigen-3 (LFA-3, CD58)
- FC receptor gamma III (CD16)
- Endotoxin binding protein receptor (CD14)

Other Receptors

- Urokinase receptor
- Folate receptor

Enzymes

- Alkaline phosphatase
- Acetylcholinesterase
- 5'-Ectonucleotidase

Other Proteins

- CD24
- CD48
- CD52 (Campath-1)
- CD66c
- CD67
- JMH-bearing protein

Clinical Findings

PNH is seen primarily in adults but has also been described in children and adolescents.²⁹ The disease displays a wide spectrum of symptoms but usually begins with an insidious onset of anemia. The degree of anemia found at diagnosis ranges from mild to severe. Despite the name, the symptom of hemoglobinuria causing a dark morning urine is often not seen. Hemolysis

TABLE 13-5 Types of Cells Observed in Paroxysmal Nocturnal Hemoglobinuria

PNH Cell Type	Sensitivity to Complement Lysis	Observed Complement Pathway Defects	GPI Protein Expression	Associated PIGA Mutations
I	Normal to near normal	Near normal lytic behavior	Near normal to mild deficiency; partial lack of DAF (CD55) and/or MIRL (CD59)	None or single point mutation
II	Intermediate (10–15 times more sensitive)	Increased C ₃ binding to cell; increased C ₃ /C ₅ convertase activity	Partial lack of DAF (CD55) and MIRL (CD59) (DAF deficiency most significant)	Missense (partial)
III	Highly sensitive (25 times more sensitive)	Increased binding of C ₃ to cell; increased C ₃ /C ₅ convertase activity; increased binding of C _{5b} complexes; increased C ₉ binding	Near total lack of DAF (CD55), MIRL (CD59), HRF	Nonsense mutation frameshift, deletion or insertion causing gene inactivation

PNH = paroxysmal nocturnal hemoglobinuria; GPI = glycophosphatidylinositol; PIGA = phosphatidylinositolglycan A; DAF = decay accelerating factor; MIRL = membrane inhibitor of reactive lysis; HRF = homologous restriction factor.

is more likely to occur in an irregular fashion, precipitated by infection, surgery, and transfusion. Chronic urinary iron loss or hemosiderinuria is a constant feature and may lead to an iron-deficiency anemia and chronic renal failure.²⁷

Abnormal platelet function in PNH patients is frequently associated with venous thromboses. As many as one-third of patients suffer significant thromboses at some time, and thrombosis is a major cause of death in these patients. Thrombotic events may cause severe abdominal or back pain or severe refractory headaches. Development hepatic vein thrombosis, mesenteric vein, or cerebral vein thromboses are most common. Thrombophlebitis may also occur, leading to pulmonary thromboembolism. Arterial thromboses are rare. Rare patients may experience bleeding due to poor platelet function.^{27,28,29}

Laboratory Evaluation

The CBC in PNH will show anemia, leukopenia, and thrombocytopenia similar to the CBC in AA. The anemia can be moderate to severe depending on the number of PNH cells and subtype(s) of cells present. Hemoglobin levels vary from normal to <6 g/dL with a median of 9.3g/dL.²⁹ The peripheral blood smear shows normochromic and normocytic cells without significant abnormal morphologies. Occasionally slight macrocytosis and polychromasia may occur owing to increased reticulocytes. (Fig. 13-11). Unlike AA, reticulocytosis is present in PNH. During episodes of severe hemolysis, an occasional nucleated red blood cell may be seen.²⁸

Because granulocytes and platelets may also have the same membrane defects, they can also be sensitive to complement lysis.³⁰ Neutropenia is present, but the cells themselves are morphologically normal. The neutrophils have decreased leukocyte alkaline phosphatase activity.³⁰ Platelet counts vary with mild to moderate thrombocytopenia. While thrombocytopenia is often present, the most frequently observed platelet dysfunction is abnormal clotting.^{29,30,31}

The bone marrow often shows erythroid hyperplasia in response to chronic intravascular hemolysis (Fig. 13-12). Over time, some patients with PNH will develop hypoplastic

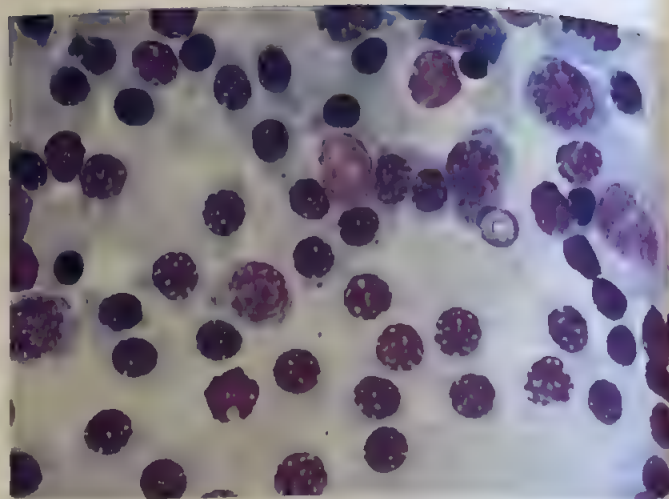


FIGURE 13-12 Bone marrow aspirate smear from a patient with paroxysmal nocturnal hemoglobinuria demonstrating erythroid hyperplasia. (Wright-Giemsa stain, $\times 500$ magnification)

or aplastic marrows. Often adequate numbers of myeloid and megakaryocytic precursors are present.

Testing for PNH includes decreased serum haptoglobin, increased plasma hemoglobin, serum indirect bilirubin, lactate dehydrogenase, hemoglobinuria, and hemosiderinuria, which indicates intravascular hemolysis.²⁷ Hemosiderinuria, a result of intravascular hemolysis, can be demonstrated by staining the patient's urine with Prussian blue. Hemoglobinuria, when present, will be detected by dipstick. This must be coupled with microscopic examination to determine that intact red cells are absent.

Historically, initial screening for PNH was done with a sugar water test (sucrose hemolysis test), which was then confirmed with the Ham's test (acidified serum lysis test) (Fig. 13-13 and Fig. 13-14). These tests have been replaced by flow cytometry testing due to the increased sensitivity. Flow cytometry utilizes monoclonal antibodies against proteins that attach to the GPI anchor proteins. Anti-CD55 and anti-CD59 are frequently used cell markers.³¹ In addition, the cells can be tested with fluorescent aerolysin,

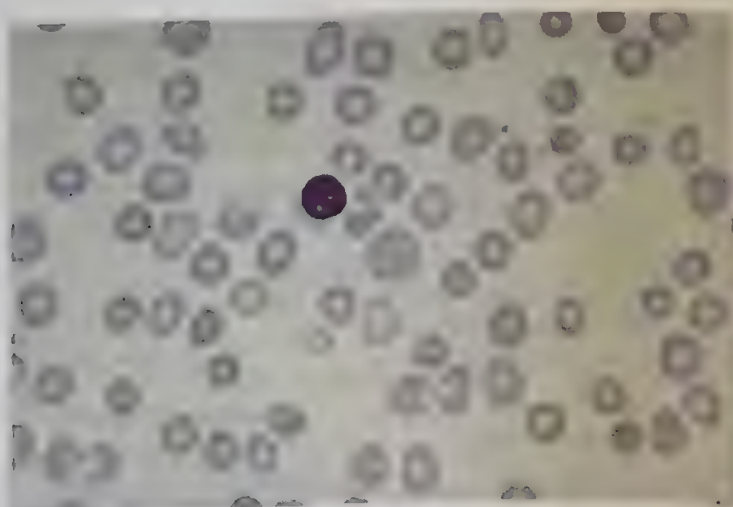


FIGURE 13-11 Peripheral blood smear from a patient with PNH (Wright-Giemsa stain, $\times 600$ magnification) demonstrating normochromic, normocytic red cells, as well as occasional hypochromic, microcytic, and polychromatophilic cells. A nucleated red cell is also seen.

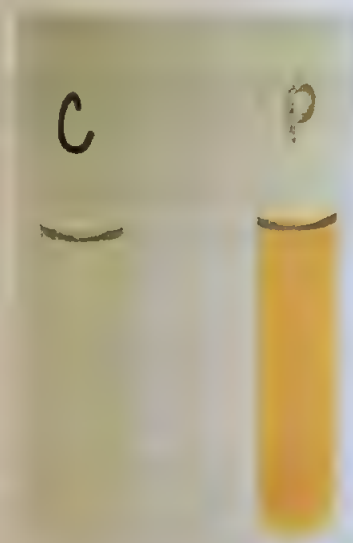


FIGURE 13-13 Sugar water test. The tube on the left represents the control (C) and the tube on the right represents the patient (P) with a positive sugar water test demonstrating 10% to 80% hemolysis associated with PNH.

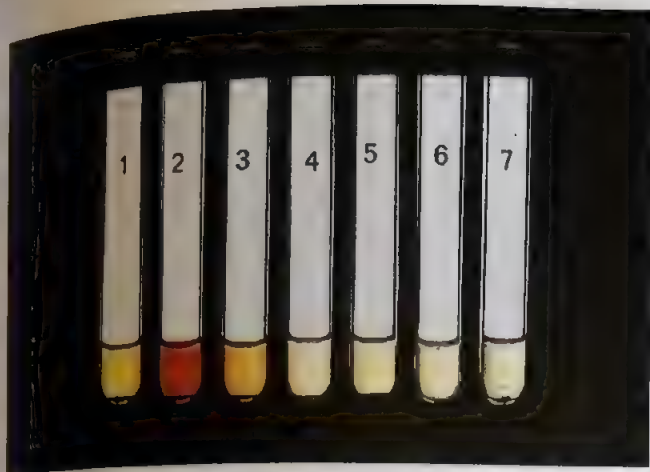


FIGURE 13-14 Ham's test. Positive results occur in patients with PNH. A positive test is reported when hemolysis occurs in tube 1, containing fresh normal serum and patient cells; tube 2, containing acidified normal serum and patient cells; and tube 3, containing acidified patient serum and patient cells.

fluorescein-tagged proaerolysin that binds to GPI anchor proteins. Detection of decreased or absent binding of fluorescent aerolysin, anti-CD55, or anti-CD59 is diagnostic of PNH.³² In most patients, populations of both normal and abnormal cells are seen, reflecting phenotypic mosaicism (Fig. 13-15).

ADVANCED CONTENT

The degree of CD55 or CD59 deficiency is often associated with the severity of disease. Three types of erythrocytes are described based on amount of GPI anchor proteins present. Type I cells have normal amounts of CD59, type II cells have decreased amounts of CD59, and type III cells lack CD59. While flow cytometry on the granulocyte and monocyte populations is technically more challenging, testing of two or more lineages of blood cells is recommended. Flow cytometry has the added advantage of being able to detect very small PNH clones (1% to 5% deficient cells) and is a sensitive method to detect early PNH, as well as emergence of PNH clones in AA. Flow cytometry is also useful after a recent hemolytic episode when the number of abnormal red cells may be decreased.^{27,31}

CRITICAL THINKING QUESTION

13-2 What are the major differences between aplastic anemia and PNH in terms of what causes the anemia?

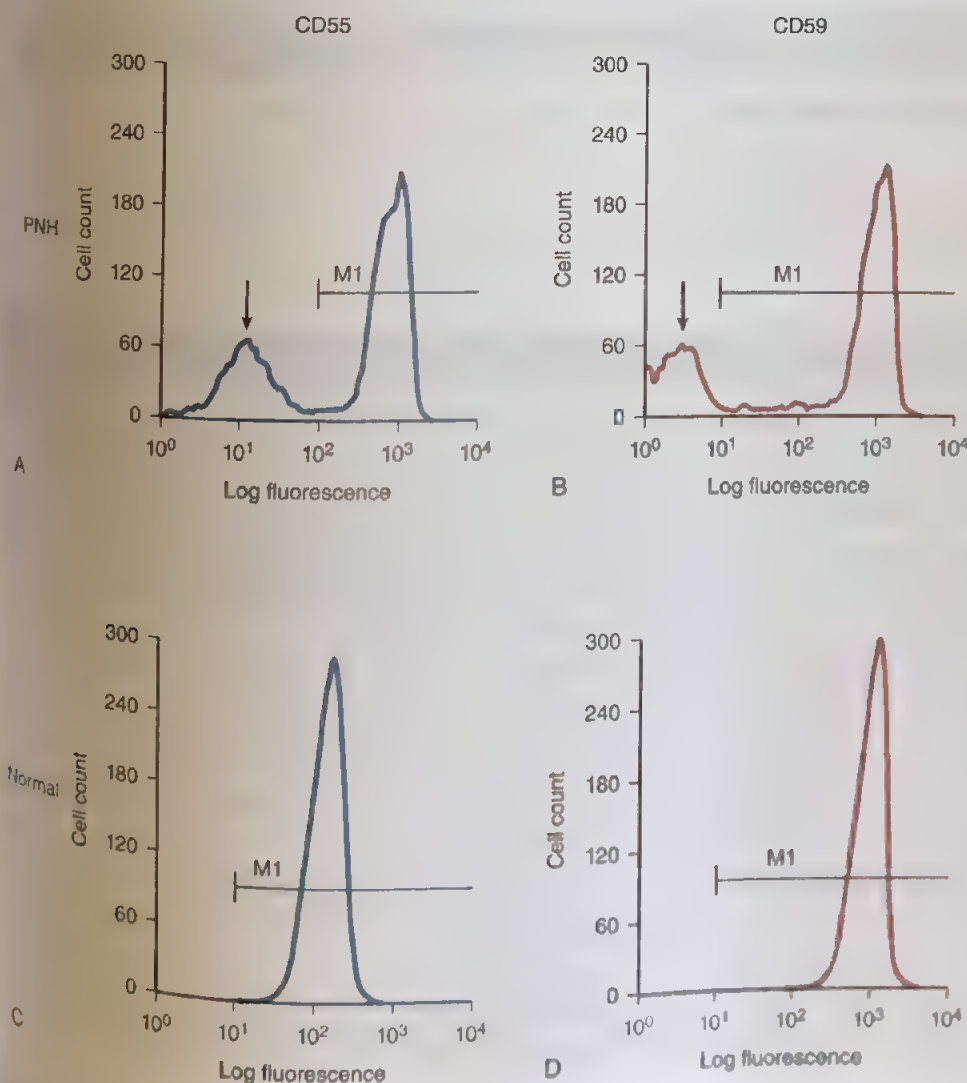


FIGURE 13-15 Flow cytometry diagnosis of PNH. The flow cytometric histograms from a patient with PNH (A, B) show decreased expression of CD55 (A, arrow) and CD59 (B, arrow) in addition to populations of red cells containing relatively normal levels of CD55 and CD59. For comparison, a normal control showing single red cell populations for both CD55 (C) and CD59 (D). (From Smith LJ. Paroxysmal nocturnal hemoglobinuria. *Curr Opin Hematol*. 2004;17:172, with permission.)

Treatment

Allogenic bone marrow transplant is considered curative but due to its risks should be limited to select patient populations.

ADVANCED CONTENT

PNH is now treated with eculizumab, a humanized monoclonal antibody that binds to C5, inhibiting its cleavage to C5a and C5b. Blocking this step in the complement cascade prevents the formation of the membrane attack complex and thus prevents cell lysis. Because eculizumab has a similar effect as a complement C5 deficiency, patients should be immunized against *Neisseria meningitidis* infection before beginning treatment. Eculizumab does not cure PNH, but it has been shown to be a safe and effective treatment. It reduces the need for blood transfusions and decreases the risk of thrombosis. However, 25% to 35% of patients continue to require occasional transfusion due to extravascular hemolysis. Eculizumab must continue to be administered indefinitely.³³

Those who have PNH with aplastic anemia or PNH with myelodysplastic disease and low bone marrow cellularity and patients on eculizumab who continue to experience serious thrombosis while on the drug may be candidates for transplant.^{27,28,32,33}

Relationships Among Conditions of Bone Marrow Hypoplasia

Aplastic anemia is a bone marrow failure disorder linked to clonal hematopoiesis. Using high-density genotyping with single nucleotide polymorphism arrays, patients with aplastic

anemia are often found to have several abnormal hematopoietic cell clones. Nearly half of aplastic anemia patients have a small clone of cells with the PNH phenotype, mutation in the *PIGA* gene, at the time of diagnosis. While gene mutations can develop with age even in normal marrow, there may be survival advantage for these and other mutated hematopoietic cells in a patient with aplastic anemia. It has been hypothesized that the PNH phenotype may allow the hematopoietic precursor to escape cytotoxic T lymphocyte destruction.³⁴ In contrast, the *PIGA* deficient clones do not have a survival advantage over normal hematopoietic cells, resulting in the mosaicism characteristically seen in patients with PNH alone.³⁵

Aplastic anemia also appears to have a connection with myelodysplastic syndrome (MDS), acute myelogenous leukemia (AML), and T-large granular lymphocytic leukemias. Regarding aplastic anemia patients treated with immunosuppression, 10% go on to develop MDS and 7% develop AML within 10 years.³⁴

Clonal expansion of T-large granular lymphocytes (T-LGL) may be found in both PNH and aplastic anemia patients. Genetic mutations associated with T-LGL and MDS can be detected in aplastic anemia patients. Thus, the underlying pathophysiology of aplastic anemia, PNH, and other bone marrow failure syndromes appear to be closely related.^{35,36}

SUMMARY CHART

- Aplastic anemia is defined as a failure of bone marrow and loss of bone marrow cellularity leading to decreased production of erythrocytes, leukocytes, and platelets, and development of peripheral blood cytopenias.
- Clinical criteria defining aplastic anemia include a bone marrow cellularity of less than 25% and at least two peripheral blood cytopenias.
- Aplastic anemia usually arises as a result of acquired damage, although rare cases are hereditary. In the majority of cases, the underlying cause of aplastic anemia is immune dysfunction.
- Of those cases of aplastic anemia in which an etiological agent can be identified, drugs, chemicals (especially benzene-type compounds), irradiation, and some infections have been implicated as causes of aplastic anemia.
- Drug-induced aplastic anemia is usually an idiosyncratic (not dose-related) reaction; the most commonly implicated drugs are chloramphenicol and phenylbutazone.
- Ionizing radiation usually causes bone marrow aplasia in an immediate dose-dependent manner, although late effects may also be seen.
- Infections and altered or autoimmune states may also cause bone marrow aplasia, probably as a result of immune destruction of the bone marrow hematopoietic precursor or stem cell.
- The congenital form of aplastic anemia, Fanconi's anemia, is characterized by an autosomal recessive inheritance pattern and progressive development of pancytopenia and bone marrow hypoplasia as well as an increased incidence of acute leukemia and other malignancies.
- Fanconi's anemia is characterized at a molecular and cytogenetic level by increased chromosomal breakage and defective DNA repair.
- Blood findings in aplastic anemia include normochromic, normocytic anemia with low numbers of reticulocytes (hypoproliferative anemia), leukopenia

SUMMARY CHART—cont'd

- (especially of myeloid and monocytic cells), and thrombocytopenia. Lymphocyte counts may be normal or decreased.
- Pure red cell aplasia is defined as a selective loss of bone marrow red cell precursors and presents clinically as an isolated hypoproliferative anemia. Causes of pure red cell aplasia include infections (parvovirus B19), reactions to drugs, and immune abnormalities. A congenital form is recognized, called Diamond-Blackfan anemia.
 - Congenital dyserythropoietic anemias (CDAs) are familial or inherited anemias that are characterized by ineffective erythropoiesis, bone marrow erythroid hyperplasia, and bizarre erythroid precursors with multiple nuclei and intranuclear chromatin bridges.
 - Paroxysmal nocturnal hemoglobinuria is an acquired hemolytic anemia resulting from a hematopoietic stem cell abnormality causing decreased levels of GPI-anchored proteins on the cell's surface. The lack of

some of these proteins leads to increased sensitivity to complement lysis of red cells.

- Diagnosis of PNH is made utilizing the flow cytometric analysis for decreased CD55 and CD59 activity
- PNH is now treated with eculizumab, an antibody that binds to C5, inhibiting its cleavage to C5a and C5b.
- There appears to be an interrelationship between aplastic anemia, PNH, and other bone marrow failure syndromes. Many patients with aplastic anemia show hematopoietic clones that have a PNH phenotype, suggesting the possibility of a pathophysiologic relationship between these disorders.
- Aplastic anemia in patients younger than 50 years of age is usually treated by bone marrow transplantation. Patients unsuitable for transplantation therapy may be treated with immunosuppressive therapy such as antithymocyte globulin and cyclosporine.

CASE STUDY 13-1

A 20-year-old woman was seen by her physician for fatigue, pallor, and easy bruising. Physical examination was unremarkable except for pallor, widespread petechiae, ecchymosis, and bleeding gums. The spleen was not enlarged. There was no history of recent exposure to drugs, toxins, or radiation. There was no history of any other illness, and she had been in good health until the past 2 weeks.

Laboratory data revealed a normochromic, normocytic anemia with a hematocrit of 20%, WBC of $20 \times 10^9/L$, and platelets of $20 \times 10^9/L$. The peripheral blood showed 9% neutrophils, 90% lymphocytes, and 1% monocytes. The corrected reticulocyte count was 0.5%. A bone marrow aspirate and biopsy specimen were markedly hypocellular, with less than 5% cellularity composed of lymphocytes and plasma cells admixed with a rare myeloid or erythroid precursor. No megakaryocytes were noted. No dysplastic changes were noted in the few hematopoietic cells seen, and there was no increase in the number of blasts. Further studies failed to identify an underlying etiology for the patient's pancytopenia.

The patient's family was tested, and she was found to have a sister who was HLA compatible, who donated peripheral stem cells for an allogeneic transplantation. Her CBC results 1 year after the transplantation showed a hematocrit of 42%, WBC of $6.0 \times 10^9/L$, and platelets of $210 \times 10^9/L$. The WBC differential showed 71% neutrophils, 21% lymphocytes, 6% monocytes, and 2% eosinophils. A bone marrow biopsy specimen showed a bone marrow cellularity of 50%, with all cell lineages present and maturing normally. The patient has had no further episodes of bleeding or infection.

QUESTIONS

1. Which initial laboratory data aligned with aplastic anemia?
2. What are possible etiologies of this disorder?
3. What is the clinical approach to therapy?

ANSWERS

1. Normocytic, normochromic anemia; low WBC, RBC, and PLT counts; low Hgb and Hct; and differential showing relatively high percentage of lymphocytes. Also, her retic count was low, not high, to indicate that her bone marrow was attempting to correct the anemia.
2. A number of causes of aplastic anemia including drugs, infections, irradiation, and chemical exposures are identified, but most cases are autoimmune in nature and are thought to arise secondary to immune destruction of bone marrow precursors. A minority of cases may be congenital. Since this patient had no history of drugs, exposure to radiation, or a recent infection, it mostly likely was an autoimmune process that caused her aplastic anemia.
3. Treatment with immune suppression or bone marrow transplant are most likely approaches. If a patient is under the age of 50 and has a bone marrow donor available, a bone marrow transplant offers the best chance for cure. In older patients and those for whom an HLA-matched donor cannot be found, immunosuppression will be used.

CASE STUDY 13-2

A 43-year-old man presented to his physician with complaints of lower back pain, fatigue, easy bruising, and a sudden onset of dark urine on arising in the morning. Laboratory studies and a bone marrow aspirate were ordered by his physician. The CBC revealed an anemia (hematocrit, 28%), leukopenia (white blood cell [WBC] count $33 \times 10^9/L$), and thrombocytopenia (platelets, $76 \times 10^9/L$). The reticulocyte count was 5.1% (3.2% corrected). The RBCs showed moderate polychromasia, slight anisocytosis, and poikilocytosis, and 1 nucleated RBC per 100 WBCs. The chemistry profile was normal except for an increased lactate dehydrogenase value and an increased indirect bilirubin. The patient's urine was positive for hemoglobin, but no red blood cells were observed on the microscopic exam. The bone marrow analysis revealed a hypercellular marrow with relative erythroid hyperplasia. There were adequate numbers of myeloid and platelet precursors.

Further studies were performed after initial test results were evaluated. Flow cytometry showed a population of red cells with decreased expression of CD55 and CD59.

The patient was immunized against *Neisseria meningitidis* and then placed on eculizumab two weeks later. The patient continued on eculizumab with improvement of his red cell count.

QUESTIONS

1. Which of the findings in this patient are inconsistent with aplastic anemia?
2. Based on all the findings, what is the most likely diagnosis?
3. What causes the destruction of red blood cells in this condition?
4. What type of hemolysis is evident in this patient? Why?
5. If the granulocytes in this patient were scored for LAP activity, what would you expect the result to be?
6. How does eculizumab prevent red blood cell destruction?

ANSWERS

1. The lack of pancytopenia is inconsistent with findings in aplastic anemia.
2. Paroxysmal nocturnal hemoglobinuria due to the hemoglobin in urine, the increase of bilirubin and LDH in the blood, and the lack of CD55 and CD59 on the red cell membranes.
3. Lack of GPI anchor proteins results in a lack of CD55 and CD59 on the red cell membranes. This allows complement to bind and hemolyze the red cells.
4. Intravascular hemolysis, because hemoglobin was present in the urine.
5. LAP would be expected to be decreased.
6. Eculizumab prevents complement activation and hemolysis of the red cells.

REVIEW QUESTIONS

1. How is aplastic anemia best defined?
 - a. A condition in which bone marrow production of red cells, white blood cells, and platelets has failed
 - b. A condition in which severe anemia is seen
 - c. A condition in which platelets are decreased
 - d. A condition in which there is pancytopenia with a hypercellular bone marrow
2. Which is the most common cause of aplastic anemia?
 - a. Drug ingestion
 - b. Toxin exposure
 - c. Immune dysfunction
 - d. Ionizing radiation
3. Which of the following causes the bone marrow destruction seen in the autoimmune form of aplastic anemia?
 - a. Production of anti-HSPC antibodies
 - b. Lack of antibody producing plasma cells
 - c. An increased population of cytotoxic T lymphocytes
 - d. Activation of natural killer lymphocytes
4. Which of the following characterizes the aplastic anemia associated with benzene exposure?
 - a. It is not related to the amount of exposure.
 - b. The anemia is usually reversible.
 - c. It always causes fatal, severe anemia.
 - d. It is usually caused by ingestion of benzene in food.
5. Which of the following statements about chloramphenicol-induced aplastic anemia is true?
 - a. The degree of bone marrow aplasia is not directly related to the dosage of the drug.
 - b. Chloramphenicol usage remains high despite its relationship to aplastic anemia.
 - c. Aplastic anemia only develops in males who use the drug.
 - d. Chloramphenicol is the only drug that has been linked to aplastic anemia.

REVIEW QUESTIONS—cont'd

6. Which of the following statements regarding ionizing radiation and aplastic anemia is true?
 - a. Damage to the bone marrow is guaranteed after exposure.
 - b. Bone marrow aplasia may develop months to years after exposure.
 - c. X-rays are safe and have not been linked to aplastic anemia.
 - d. Once the radiation has been removed, the aplastic anemia will go away.
7. What is the most common congenital disorder associated with aplastic anemia?
 - a. Fanconi's anemia
 - b. Thrombocytopenia-absent radius (TAR) syndrome
 - c. Congenital dyserythropoietic anemia, type 1
 - d. Diamond-Blackfan anemia
8. Which of the following statements regarding the clinical features of aplastic anemia is true?
 - a. Symptoms usually develop suddenly
 - b. Patients experience anxiety, nervousness, and vigor
 - c. Petechiae, purpura, and ecchymoses may be present
 - d. Signs of infection are never present
9. Which of the following is seen in the peripheral blood of patients with aplastic anemia?
 - a. Hypochromic, normocytic anemia
 - b. Increased reticulocyte count
 - c. Relative lymphocytosis
 - d. Increased granulocytes
10. What is the appearance of the bone marrow in aplastic anemia?
 - a. Hypercellular
 - b. Normocellular
 - c. Hypocellular
 - d. Fibrotic
11. What is the treatment of choice for severe aplastic anemia in patients who are younger than age 50?
 - a. Multiple transfusions
 - b. Androgens
 - c. Bone marrow transplantation
 - d. EPO therapy
12. What is the definition of pure red cell aplasia?
 - a. Lack of lymphoid precursors with normal erythroid precursors in the bone marrow
 - b. Abnormal, giant normoblasts in the bone marrow
 - c. Lack of erythroid precursors with normal white blood cell and megakaryocytic precursors
 - d. Dysplastic red cell precursors with normal white cell and megakaryocytic precursors
13. What features do the congenital dyserythropoietic anemias (CDAs) have in common?
 - a. Anemia, microcytosis, erythroid hyperplasia, and abnormal erythroblasts
 - b. Anemia, erythroid hyperplasia with abnormal erythroblasts, and indirect hyperbilirubinemia
 - c. Anemia, lysis in acidified serum, and indirect hyperbilirubinemia
 - d. Anemia, macrocytosis, erythroid hyperplasia with abnormal erythroblasts, and indirect hyperbilirubinemia
14. Which statement best describes paroxysmal nocturnal hemoglobinuria?
 - a. Acquired anemia associated with complement mediated hemolysis
 - b. Congenital hemolytic anemia associated with the inflammatory response
 - c. A premalignant condition that almost always results in development of acute leukemia
 - d. A common disorder that frequently resolves with time
15. What causes the red cell defect of PNH?
 - a. Rare red cell antigens
 - b. Lack of GPI-anchored proteins on the erythrocyte membrane
 - c. Excessive amounts of complement components C5 to C9
 - d. Glucose-6-phosphate dehydrogenase enzyme deficiency
16. Which of the following is the basis of the flow cytometric test for diagnosis of PNH?
 - a. PNH will have increased amounts of complement detected on the cell surface.
 - b. PNH cells are easily lysed and will show decreased number when analyzed.
 - c. A subset of the patient cells will show decreased levels of CD55 and CD59.
 - d. All of the patient cells will show decreased levels of ABO antigens on the membrane.
17. How are most patients with paroxysmal nocturnal hemoglobinuria treated?
 - a. Eculizumab, a drug that inhibits cleavage of C5 to C5b
 - b. Monthly transfusion of platelets and granulocytes
 - c. Use of immunosuppressive drugs like antithymocyte globulin (ATG)
 - d. Weekly doses of EPO
18. What is the major genetic mutation seen in many patients with aplastic anemia?
 - a. Lack of GPI-AP
 - b. *PIGA* mutation
 - c. Beta globulin mutation
 - d. *PKLR* mutation

See answers at the back of this book.

Hemolytic Anemias

Extracorpuscular Defects

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CHAPTER OUTLINE

Introduction

Immune Hemolytic Anemia

Immune Hemolysis

Classification of Immune Hemolytic Anemia

Nonimmune Hemolytic Anemia

Intracellular Infections

Extracellular Infections

Mechanical Etiologies

Chemical and Physical Agents

Acquired Membrane Disorders

Summary Chart

Case Study 14-1

Case Study 14-2

Case Study 14-3

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 14-1 Define hemolytic anemia and list mechanisms of immune hemolysis.
- 14-2 Define alloimmune hemolytic anemia.
- 14-3 Differentiate acute from delayed hemolytic transfusion reactions.
- 14-4 Describe the pathophysiology and causes of hemolytic disease of the fetus and newborn.
- 14-5 Define autoimmune hemolytic anemia.

- 14-6 Compare and contrast warm autoimmune hemolytic anemia with cold agglutinin disease and paroxysmal cold hemoglobinuria.
- 14-7 List four mechanisms of drug-induced hemolytic anemia.
- 14-8 Differentiate intracellular causes of hemolytic anemia based on laboratory findings and clinical presentation.
- 14-9 Describe mechanical causes of hemolytic anemia.
- 14-10 List different chemical causes for hemolytic anemia.

Hemolytic anemias are associated with the shortened survival of the erythrocyte and lead to a decrease in the red blood cell's capacity to deliver oxygen to the tissues. Hemolytic anemias can be divided into two classifications: those caused by an immune process, and those that are non-immune mediated. This chapter describes in detail the mechanisms of immune hemolysis, including both autoimmune and alloimmune hemolytic anemias. In addition, nonimmune hemolytic anemias associated with infections and those arising from mechanical, chemical, and physical hemolysis, as well as acquired membrane disorders, are discussed.

Immune Hemolytic Anemia

The term **immune hemolytic anemia** describes a group of disorders in which erythrocytes are destroyed prematurely by an immune-mediated process. This condition can occur as either an intravascular or an extravascular process. In **intravascular hemolysis**, red blood cells are destroyed within the vascular system. In **extravascular hemolysis**, they are destroyed outside the vascular system in the mononuclear phagocyte system (primarily in the spleen and liver).

Immune Hemolysis

Immune hemolysis results from antibodies, complement, or both attaching to the red blood cell membrane, and its diagnosis is confirmed by a positive direct antiglobulin test (DAT).

Role of Complement

Complement is a group of serum proteins that interact with each other to bring about, among other events, complement-dependent cell lysis. Complement can be activated by three different routes: the classical pathway, the alternative (properdin) pathway, or the lectin (MBL) pathway.¹

▶ ADVANCED CONTENT

Classical Pathway

Activation of the classical pathway is initiated by immune complexes containing immunoglobulin G (IgG1, IgG2, and IgG3) or IgM. The first complement component, C1, consists of three subunits—C1q, C1r, and C1s—as well as calcium (recognition unit). C1q initiates the complement

cascade by interacting with the FC portion of the immunoglobulin (Fig. 14-1). Clq then causes the activation of Clr, which then activates Cls. (A bar across the top of a complement component denotes its active form as shown in Figures 14-1 and 14-2.) C4 is the second complement protein to be activated. This occurs when Cls cleaves C4 into its activated components, C4a, which remains in the plasma, and C4b, of which a small number of molecules attach to the cell membrane, with the rest remaining in the plasma in the inactive form. C2 attaches to C4b in the presence of magnesium and is then cleaved by Cls into a and b subunits. C4b2a combines with C4b and forms the enzyme C3 convertase (C4b2a), and C2b is released into the plasma.

Amplification of complement activity now occurs with the action of C3 convertase on C3. C3 convertase (C4b2a) cleaves C3 into its active components, C3a and C3b, and is able to cleave hundreds of C3 molecules. C3a is released into the plasma and acts as an anaphylatoxin. C3b binds to the cell membrane and combines with C4b2a to form another enzyme, C5 convertase (C4b2a3b). Some of the C3b molecules attach to other sites on the cell, are inactivated (iC3b), or are cleaved by C3 inactivator to C3c, which

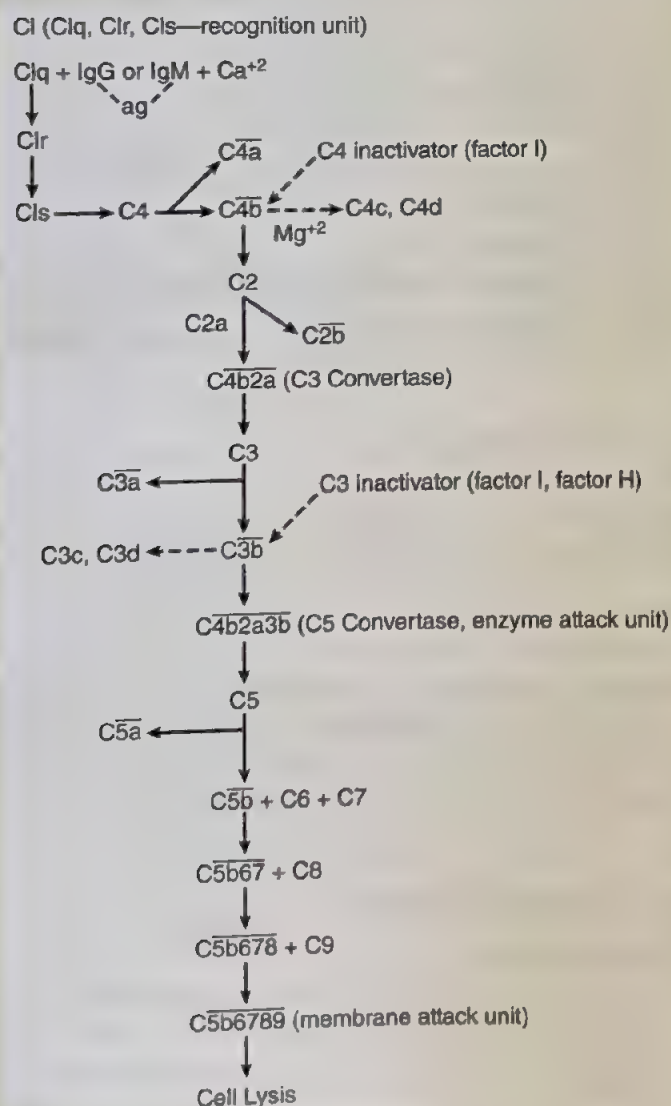


FIGURE 14-1 Classical pathway of complement activation.

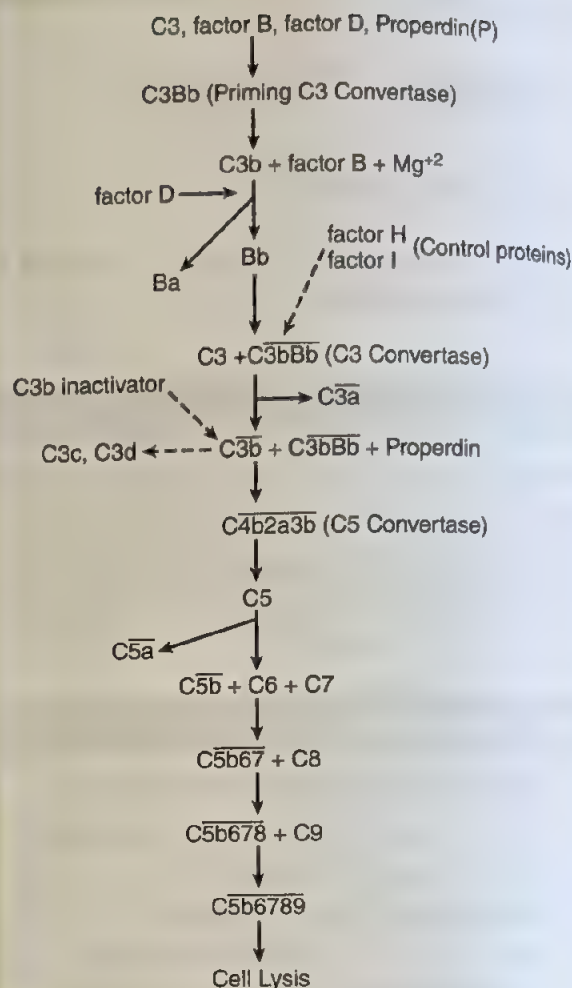


FIGURE 14-2 Alternate pathway of complement activation.

is released into the plasma, and to C3d, an inactive subunit that remains attached to the cell. The components C4, C2, and C3 are referred to as the *enzyme activation unit*.

C5 convertase (C4b2a3b) cleaves C5 into the components C5a, which is released into the plasma and acts as an anaphylatoxin and a chemotactic agent, and C5b, which binds C6 and C7 to the cell membrane. Membrane-bound C5b67 causes binding of C8, resulting in immediate ion flux into the cell and the beginning of cell lysis. The C5b678 complex can bind several C9 molecules, together forming the membrane attack unit, C5b6789, which causes cell lysis and accelerated movement of ions into the cell. With the binding of C9, the rate of cell lysis is greatly accelerated (see Fig. 14-1).

Complement activity is regulated by certain inhibitors (Cls inhibitor, C3b inactivator, C4 inactivator) and by the instability of certain components (C4b2a, and C4b2a3b).

Alternative (Properdin) Pathway The alternative, or properdin, pathway of complement activation also results in cell lysis but by a different mechanism and group of proteins. The alternative pathway bypasses the complement components C1, C2, and C4, and begins with the spontaneous hydrolytic cleavage of C3. This pathway consists of a distinct group of proteins: complement component C3; a distinct group of proteins: complement component C3; factor B, which is enzymatically cleaved into fragments Bb (biologically active) and Ba; factor D, which cleaves factor

B; properdin (P), a serum protein that stabilizes the C3bBb complex; and factor H (C3b inactivator/accelerator), which aids in controlling activation of the alternative pathway² (see Fig. 14-2).

The alternative pathway may be triggered by the interaction of C3b with certain microorganisms, polysaccharides, lipopolysaccharides, aggregates of IgA, and cells or particles even in the absence of specific antibody. Present in the plasma are small amounts of a "priming" C3 convertase (C3bBb). The priming C3 convertase is produced continuously, owing to spontaneous interaction of intact C3, factor B, factor D, and properdin, an event not requiring activating substances. This results in the formation of small amounts of C3b. C3b binds to the cell surface and, under appropriate conditions and in the presence of magnesium, causes the attachment of factor B. The bound factor B is cleaved by factor D, releasing the Ba fragment and uncovering the C3 cleaving site on the Bb fragment. The C3bBb complex can rapidly lose activity or disassociate unless properdin is present. Properdin binds to the C3b part of the complex and stabilizes it. The C3bBbP fragment (C3 convertase) then cleaves more C3, resulting in C3a and C3b fragments. A complex of C3bBb3bP (C5 convertase) forms and cleaves C5 into its fragments, C5a and C5b. C5b, together with C6, C7, C8, and C9, form the membrane attack unit, the same way as in the classical pathway, which results in cell lysis (see Fig. 14-2).

Control mechanisms also exist in the alternate pathway, just as they do in the classical pathway. Spontaneous dissolution of C3bBb may occur, or factor H with factor I protein may compete with factor B, as may Bb for the C3b fragment, then blocking the formation of C3 convertase (C3bBb). Factor I degrades C3b and C4b, aided by cofactors. Factor H has cofactor activity, binds to C3b, and has decay accelerating activity of the C3 and C5 convertases.²

Lectin (MBL) Pathway The lectin pathway can be triggered when mannose-binding lectin (MBL) attaches to mannose on microbial cell walls. Once this occurs, the subsequent cascade of reactions is the same as the classical complement pathway.

Mechanisms of Immune Hemolysis

Intravascular Hemolysis Intravascular hemolysis, as the name implies, occurs within the vascular system and results from activation of the classical complement pathway via IgM or IgG antibodies (see Chapter 23 for an explanation of immunoglobulins). Intravascular hemolysis occurs when antibodies bind to antigenic determinants on red cells and activate complement to completion (i.e., lysis). This occurs only when the activation process is intense enough to overwhelm the natural regulatory process that inactivates complement. IgM is a very efficient activator of complement because of its pentameric structure. A single molecule of IgM is capable of initiating complement activation by the classical pathway.^{3,4} The ability of IgG antibodies to activate complement is dependent on the following factors:

1. The IgG subclass; IgG3 is the most efficient at activating complement, followed by IgG1, then IgG2. IgG4 is not

capable of complement activation.⁵ The biological properties of the IgG subclasses are summarized in Table 14-1.

2. The number and location of IgG molecules on the red cell surface; at least two IgG molecules must be in close enough proximity to allow cross-linking of complement receptors, which initiates complement activation.^{3,5}
3. The physical location of the red cell antigens influences the binding of IgG.
4. The ability of the immunoglobulin to remain attached to the red cell surface (avidity) is important as well.

Antibody-Dependent Cellular Cytotoxicity

Another possible mechanism of direct (intravascular) lysis of immunoglobulin-coated red cells is antibody-dependent cellular cytotoxicity (ADCC). Many white blood cell lines (macrophages/monocytes, neutrophils, and natural killer lymphocytes [NK cells]) have receptors on their membranes that bind immunoglobulins and complement degradation products.⁶ The cells with such receptors are collectively referred to as **effector cells**. The effector cell receptors specific for IgG1 or IgG3 are called FC receptors (FcR) because they bind the FC portion of these immunoglobulins (see Chapter 23 for a review of immunoglobulin structure). These effector cells also have receptors for complement degradation products, C3b and iC3b. These complement receptors (CR) are called CR1 and CR3, respectively. ADCC results when the immunoprotein (IgG3, IgG1, C3b, or iC3b) is bound to its respective FcR or CR of the effector cell. This interaction causes the effector cell to release lytic enzymes that lyse the target cell covered in antibody and complement.⁷ It should be noted that not all IgG1 and IgG3 immunoglobulins are capable of mediating lysis.⁸ Complement degradation products appear to work synergistically with IgG3, IgG1, or both to enhance ADCC.^{7,9,10}

Laboratory Findings

Laboratory findings associated with all hemolytic anemia include the presence of anemia (commonly normocytic but occasionally macrocytic); reticulocytosis (increased reticulocyte count), reflecting erythroid hyperplasia of the bone marrow; and accumulation of the products of red cell catabolism. Patients will show increased serum bilirubin (primarily indirect or unconjugated but also including direct or conjugated) and elevated lactate dehydrogenase (LD), primarily isoenzyme LD-1. In immune hemolytic anemia, the DAT will be positive. (See under Extravascular Immune Hemolysis

TABLE 14-1. Biological Properties of IgG Isotypes

Characteristic	IgG1	IgG2	IgG3	IgG4
% Total serum IgG	65-70	23-28	4-7	3-4
Complement fixation (classic pathway)	Yes	Yes	Yes	No
Binding to macrophage FC receptors	Yes	No	Yes	No
Placental transfer	Yes	Yes	Yes	Yes
Biological half-life (days)	21	21	7-8	21

below for a discussion of the DAT.) The laboratory findings in immune hemolysis, as well as mechanisms and organ involvement, are summarized in Table 14-2. (For a review of hemoglobin catabolism, see Chapter 2, Erythrocyte Senescence.)

Hemoglobin is released into the blood when red cells are destroyed intravascularly. This condition of free hemoglobin liberation in the blood is called **hemoglobinemia**. Free hemoglobin is filtered through the kidneys, resulting in **hemoglobinuria** (free hemoglobin in the urine). Hemoglobinuria may be confused with **hematuria** (intact red cells in the urine), especially when the urine is red. It is important to distinguish between the two because hemoglobinuria is an indicator of hemolysis, whereas hematuria is not related to hemolysis. Microscopic examination of the urine may be helpful in identifying intact red cells to distinguish hematuria from hemoglobinuria. In chronic intravascular hemolysis, hemosiderin may appear in the urine (hemosiderinuria).

In hemolysis, free hemoglobin in the blood is bound by a molecule called **haptoglobin**, which is consumed in the process. Within hours of intravascular hemolysis, haptoglobin is depleted. As little as 5 mL of lysed red cells can bind all of the available haptoglobin. However, decreased haptoglobin is not specific for intravascular hemolysis since it is also seen in extravascular hemolysis. In addition, haptoglobin is rapidly synthesized in the liver and can return to normal levels within 24 hours. Because haptoglobin is an acute phase reactant protein, concentrations may vary considerably, depending on several factors, such as underlying disease processes. Consequently, care must be taken when interpreting haptoglobin levels as an indicator of hemolysis. It is advisable to have a "baseline" haptoglobin level to compare to the haptoglobin level following suspected hemolysis. See Figure 14-3 for the sequence of laboratory findings.

Extravascular Immune Hemolysis Extravascular hemolysis is the phagocytosis of red cells by fixed phagocytes within the mononuclear phagocyte system (MPS), formerly called the reticuloendothelial system. The two major organs of the MPS are the spleen and the liver. In the red pulp region of the spleen, macrophages with FC receptors (see ADCC, earlier) line the splenic cords. Antibody-coated red cells interact

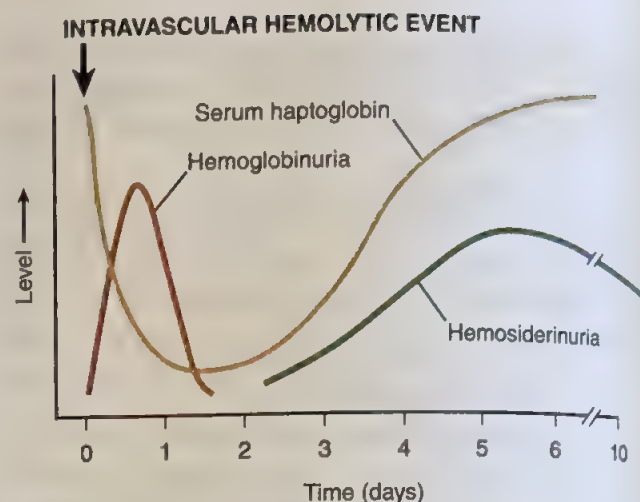


FIGURE 14-3 Indicators of acute intravascular hemolysis. Within a few hours of an acute hemolytic event, free hemoglobin is cleared from plasma and the serum haptoglobin falls to undetectable levels; hemoglobinuria ceases soon after. If no further hemolysis occurs, the serum haptoglobin level recovers, and methemalbumin disappears within several days. The urinary hemosiderin can provide more lasting evidence of the hemolytic event.

with the FC receptors, resulting in complete or partial phagocytosis. In the case of partial phagocytosis, part of the red cell membrane is removed. If the red cell membrane is able to repair itself, the normal biconcave disk is converted to a sphere-shaped red cell called a spherocyte. Spherocytes lack deformability and become physically trapped in the spleen; those that do escape the spleen can be seen in the peripheral blood, and their presence is indicative of immune-mediated hemolysis (Fig. 14-4).

Activation of Extravascular Hemolysis

As previously mentioned, both IgG and IgM antibodies are capable of activating complement. However, the activation process does not always go to completion (C1 through C9). In most cases, complement activation is stopped by an inhibitory factor (control mechanism) at the C3b stage. C3b is cleaved to form iC3b. If activation is stopped, iC3b is further broken down into C3d, which then remains attached to the red cell membrane.⁸

TABLE 14-2 Mechanisms of Immune Hemolysis

	Intravascular	Extravascular
Mechanisms	<ul style="list-style-type: none"> IgM or IgG3, IgG1, IgG2 (two IgG molecules within close proximity of each other) activate complement to completion Antibody-dependent cellular cytotoxicity (ADCC) 	<ul style="list-style-type: none"> IgM and/or IgG sensitization with/without iC3b (inactivated complement) Cell-mediated phagocytosis
Organ Involvement	Occurs within blood vessels	<ul style="list-style-type: none"> Spleen: IgG alone or IgG + iC3b-coated cells Liver: iC3b alone or IgG + iC3b-coated cells
Laboratory findings	<ul style="list-style-type: none"> Hemoglobinemia Hemoglobinuria Serum haptoglobin: marked decrease Indirect bilirubin: elevated Lactate dehydrogenase (LD): elevated Positive direct antiglobulin test 	<ul style="list-style-type: none"> Spherocytosis Serum haptoglobin: decreased Indirect bilirubin: elevated Lactate dehydrogenase (LD): elevated Positive direct antiglobulin test

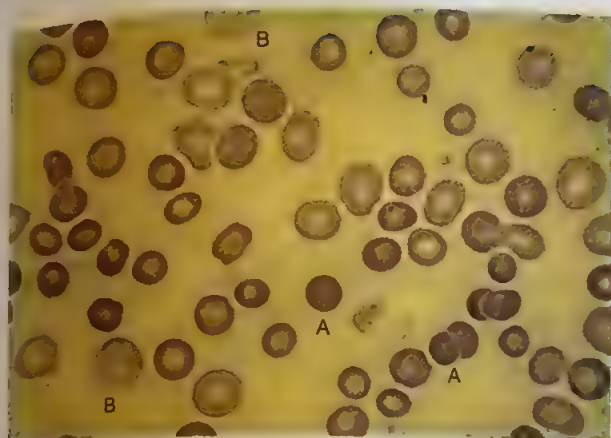


FIGURE 14-4 Autoimmune hemolytic anemia (peripheral blood). Note (A) spherocytes and (B) polychromasia.

ADVANCED CONTENT

Red cells coated with IgG1 or IgG3 are preferentially removed in the spleen⁶ rather than the liver, because blood passing through the spleen becomes hemoconcentrated, altering the ratio between free IgG in the plasma and cell-bound IgG. Free IgG can bind to FC receptors, blocking their ability to bind the IgG attached to red cells. The condition of hemoconcentration in the spleen shifts the ratio of free IgG to red cell-bound IgG in favor of the red cell-bound IgG. The activated form of complement (C3b) or its inactivated form (iC3b) are not present in the free form in plasma; therefore, the hemoconcentration of blood in the spleen does not contribute significantly to the destruction of complement-coated red cells.⁹ However, cells coated with both IgG and C3b/iC3b are phagocytized more efficiently in the spleen than if they are coated by IgG alone.⁹

The liver has the largest concentration of macrophages with receptors specific for immune complexes; thus, the liver is the major site of removal for red cells coated with complement or heavily coated with IgG.⁴ There is very little removal of red cells coated with small amounts of IgG in the liver, because of the high concentration of free IgG located there. Cells sensitized with both IgG and iC3b/C3d are removed in the liver and spleen.

Laboratory Findings

Spherocytes are commonly seen on the peripheral blood smear when extravascular immune hemolysis has occurred. Serum bilirubin (primarily indirect) and LD are usually elevated, and urobilinogen may be increased in urine and stool specimens. The DAT is usually positive. The DAT (also called the **direct antiglobulin test**, and previously called the **direct Coombs' test**) detects antibody and/or complement coating patient red cells. In the DAT, patient red cells are washed to remove adherent proteins and then reacted with reagent containing high-titer, polyspecific antibodies against both IgG (all subclasses) and complement, then examined for resulting red cell agglutination; this is called the **polyspecific DAT**. If this test is positive,

testing is repeated with monospecific reagents that recognize IgG and complement (C3d or C3b + C3d) separately to determine which is involved.¹⁰ The factors that influence the presence and extent of immune hemolysis are listed in Box 14-1.

CRITICAL THINKING QUESTION

14-1 Decreased red blood cell counts and the presence of reticulocytes can be seen in many different anemic states. Why do increased levels of bilirubin and LD, along with a positive DAT, contribute specific findings related to hemolytic anemia caused by an immune process?

See answers to all Critical Thinking Questions at the back of this book.

Classification of Immune Hemolytic Anemia

Numerous classifications of immune hemolytic anemias have been proposed; however, three broad categories are usually used:

1. **Alloimmune:** The patient produces alloantibodies to foreign red cell antigens introduced through transfusions, pregnancy, or organ transplantation.
2. **Autoimmune:** The control mechanism preventing autoreactive antibodies is lost and antibodies directed against the patient's own red cells develop.
3. **Drug-induced:** The patient produces antibodies directed at a particular drug, its metabolites, or red cells coated with the drug. These antibodies then destroy red cells.

Alloimmune Hemolytic Anemia

Alloimmunization is the process in which the immune system of an individual is stimulated by a foreign antigen and produces the corresponding antibody. The antibody produced

BOX 14-1 Factors Influencing the Presence and Extent of Immune Hemolysis

Antibody

Immunoglobulin class and subclass

Concentration

Avidity

Thermal reactivity (determined by the nature of the predominant noncovalent bonds formed at the time of the antigen-antibody reaction)

Antigen

Number and density

Cellular distribution

Presence of soluble antigen

Complement

Concentration of complement factors

Concentration and activity of regulating factors

Mononuclear Phagocytic System

Activity of phagocytic cells (influenced by underlying disease processes, generation and activity of lymphokines and interleukins, and any concurrent drug therapy)

by this immune response is termed an **alloantibody**. The antibody coats the foreign red cells introduced into the circulation, resulting in shortened red cell survival. Alloantibody production can result from:

1. Transfusion of blood (antibodies are produced against foreign donor red cell antigens)
2. Pregnancy (antibodies are produced against "foreign" antigens on fetal cells released into the maternal circulation)
3. Organ transplantation (antibodies are produced against foreign antigens on the transplanted organ or "passenger cells" that may be released into the recipient).

Alloimmune hemolytic anemia is usually associated with blood transfusions. An antibody present in the recipient is directed against a foreign red cell antigen located on the transfused cells. This antibody destroys transfused red cells but not native red cells. Basically, there are two types of transfusion reactions: acute hemolytic and delayed hemolytic.

Acute Hemolytic Transfusion Reactions Acute hemolytic transfusion reactions (acute HTRs) are characterized by acute intravascular hemolysis and are associated with the ABO blood group antibodies. The immunoglobulins associated with the ABO blood group are IgM, IgG, and IgA. In group A and B individuals, the majority of antibody is IgM with a minor amount of IgG.¹¹ Group O individuals produce anti-A, anti-B, and anti-A,B antibodies. The anti-A,B antibody is usually IgG in nature.¹¹ Ordinarily, an individual possesses naturally stimulated antibodies directed toward the A and/or B antigens absent from their own red cells. As previously stated, IgM antibodies are efficient activators of complement, which results in immediate destruction of the transfused cells, because these antibodies are naturally stimulated and are already present in the serum.

Symptoms of acute HTRs are variable. Typical symptoms can include fever, shaking, chills, and pain or a burning sensation along the infusion site. Other symptoms include nausea, vomiting, lower back pain, hypotension, and chest pain. Because of the risk of severe transfusion reactions, it is crucial for the patient's pretransfusion vital signs to be recorded, and the patient must be continuously monitored throughout the transfusion. See Box 14-2 for a list of clinical features.

The laboratory findings in acute HTRs are those associated with intravascular hemolysis (see Table 14-2). The treatment of acute HTR focuses on prompt termination of the transfusion and treatment of any signs or symptoms of shock with supportive measures. Stroma from hemolyzed red cells can clog and damage renal glomeruli; thus, intravenous fluids are administered to maintain renal function.

Delayed Hemolytic Transfusion Reactions Delayed hemolytic transfusion reactions (delayed HTR) are associated with antibodies to blood groups other than the ABO blood group. Antibodies implicated in delayed HTR are usually IgG, which may activate complement, causing sensitization of the red cells with C3 but seldom leading to intravascular hemolysis. Delayed HTRs are the most common type of transfusion reactions. They are caused by an anamnestic or

BOX 14-2 Clinical Features of Acute Hemolytic Transfusion Reactions

- Fever
- Hemoglobinuria
- Chills
- Shock
- Chest pain
- Generalized bleeding
- Hypotension
- Oliguria
- Nausea
- Anuria
- Flushing
- Back pain
- Dyspnea
- Pain at infusion site

secondary immune response to the transfused red cells. This occurs in previously immunized patients whose alloantibody level (after the initial stimulation) has dropped to serologically undetectable levels. As a result, the initial antibody screening and compatibility tests on the patient's pretransfusion sample are negative. When the patient is reexposed to the foreign red cell antigen (transfused), an anamnestic response is mounted by the recipient. The titer rises and the antibody may be detected in the posttransfusion sample as early as 48 hours after the transfusion. This type of reaction is termed *delayed* because it takes time for the patient to produce sufficient antibody to destroy the transfused cells. Characteristically the reaction may occur anywhere from 2 to 10 days after transfusion.¹²

The symptoms of delayed HTRs are usually mild and nonspecific; therefore, delayed HTRs may not be recognized by clinicians.¹³ Symptoms include mild fever, mild jaundice, and an unexpected fall in hemoglobin (or conversely, lack of expected rise in hemoglobin after transfusion). Laboratory findings are those associated with extravascular hemolysis, but most cases are subclinical and discovered only serologically (through direct antiglobulin test, antibody screening, and compatibility tests).¹³

Treatment is rarely necessary, and investigation focuses on accurately identifying the antibody to ensure blood for future transfusions will be negative for the foreign antigen that corresponds to the patient's antibody. Some antibody specificities, such as anti-D, are capable of causing both acute and delayed hemolytic transfusion reactions. The antibodies most commonly implicated in delayed HTRs are listed in Table 14-3, and the laboratory findings are summarized in Table 14-4.

CRITICAL THINKING QUESTION

- 14-2** Why do antibodies from ABO blood groups trigger acute hemolytic transfusion reactions, whereas antibodies from other blood groups often do not?

TABLE 14-3 Antibodies Most Commonly Implicated in Delayed Hemolytic Transfusion Reactions (DHTRs)

Antibody	Blood Group System
Anti-Jk ^a	Kidd
Anti-K	Kell
Anti-c	Rh
Anti-E	Rh
Anti-Fy ^a	Duffy
Anti-Jk ^b	Kidd
Anti-C	Rh
Anti-e	Rh

TABLE 14-4 Laboratory Findings: Differential Diagnosis of Hemolytic Anemia

Parameter/Analyte	Extravascular	Intravascular
Serial hemoglobin and hematocrit	Decreased	Decreased
Reticulocyte count*	Increased	Increased
RBC morphology	Spherocytes	
Serum bilirubin (indirect)	Increased	Increased
Serum haptoglobin [†]	Decreased*	Decreased
Serum LD (isoenzyme LD 1) [‡]	Increased	Increased
Hemoglobinemia	Absent	Present
Hemoglobinuria	Absent	Present

*May not show increase immediately.

[†]Important to compare with a prehemolysis "baseline" value.[‡]More likely to be markedly decreased with intravascular hemolysis.

LD = lactate dehydrogenase.

Hemolytic Disease of the Fetus and Newborn Hemolytic disease of the fetus and newborn (HDFN) is an immune hemolytic disorder in which fetal or neonatal red cells are destroyed by maternal alloantibodies. In HDFN, maternal-fetal blood group incompatibility is always present. Maternal IgG antibodies directed against "foreign" antigens on fetal red cells cross the placenta and destroy the red cells in the fetal circulation. Only IgG antibodies can cross the placenta; IgM cannot and does not cause HDFN.

Pregnant women can become immunized to "foreign" fetal red cell antigens when fetal blood enters the maternal circulation during pregnancy or at delivery (fetal-maternal hemorrhage). The amount of whole blood exchanged is normally only 1 mL. There is some evidence that as little as 0.03 mL of red cells can stimulate an immune response.⁹ Thus, it is possible to become immunized from miscarriages and abortions. Blood transfusions can also stimulate an immune response, as mentioned earlier in the discussion of alloimmune hemolytic anemia. If a pregnant woman has been previously immunized to a foreign red cell antigen and that antigen is present on the

red cells of the fetus, the exchange of blood that occurs early in the pregnancy will be sufficient to stimulate an anamnestic response in the mother. The mother can produce increasing amounts of antibody directed against the fetal red cells.¹⁴ The fetal red cells will become coated with maternal antibodies and destroyed by extravascular hemolysis. Responding to this increased red cell destruction, fetal hematopoietic tissue increases erythrocyte production. The fetal bone marrow may not be able to keep up with the increased need for red cells. Extramedullary hematopoiesis is expanded, causing liver and splenic enlargement. The fetus may not be able to compensate for the hemolysis, and an anemia characterized by increased numbers of nucleated RBCs (erythroblasts) in peripheral blood may result. Hence, the term *erythroblastosis fetalis* has been used to describe HDFN. There are two major forms of HDFN: that associated with ABO and that associated with Rh(D) or other blood group antigens. Table 14-5 lists the frequencies of the various types of HDFN, and Table 14-6 provides a comparison of ABO and Rh(D) HDFN.

Autoimmune Hemolytic Anemia

Autoimmune hemolytic anemia (AIHA) represents an abnormality within the immune system whereby the ability for self-recognition of an individual's own red cell antigens is lost. Recent experiments support that recognition of self occurs during embryogenesis by inactivation of autoreactive B and T lymphocytes. Under certain conditions (e.g., bacterial or viral infections), these autoreactive B and T lymphocytes escape the mechanism for tolerance of self.¹⁵ As a result, patients produce antibodies that bind to their own red cells (autoantibody). AIHA can be broadly divided into warm or cold types. The warm type (WAIHA) is the most common, accounting for approximately 70% of all cases.⁹ This type involves autoantibodies whose serological reactivity is optimal at 37°C. Cold AIHA involves autoantibodies whose serological reactivity is optimal at 4°C but that also react at temperatures between 25°C and 31°C. Two types of cold AIHA have been described: Cold agglutinin syndrome (CAS) and Paroxysmal cold hemoglobinuria (PCH).

Some drugs may induce the formation of autoantibodies that may be difficult to distinguish from other cases of AIHA. Drug-induced immune hemolytic anemia is the third type of AIHA, representing approximately 12% of cases in various studies.⁹ The frequency of the various types of AIHA is listed in Table 14-7.

Warm Autoimmune Hemolytic Anemia Warm autoimmune hemolytic anemia (WAIHA) is one of the most common causes of hemolytic anemia in adults, with a slightly higher

TABLE 14-5 Frequency of Types of Hemolytic Disease of the Fetus and Newborn

Disease Type	Frequency
ABO HDFN	65%
Rh HDFN	33%
Other	2%

TABLE 14-6 Comparison of ABO and Rh Hemolytic Disease of the Fetus and Newborn

Characteristic	ABO	Rh
Severity	Mild	Severe
Child affected	First-born (40%–50% of cases)	Usually second or subsequent births (first-born: 5% of cases)
Blood groups	Mother: O	Mother: Rh-negative
	Child: A or B	Child: Rh positive
Anemia	Uncommon, mild	Severe
Stillbirths/hydrops fetalis	Rare	Frequent
Jaundice	Mild	Severe
Spherocytes on peripheral smear	Usually present	Absent
Direct antiglobulin test (DAT) result	Negative or weakly positive	Positive
Maternal antibodies	Inconsistent, inconclusive	Always present
Antenatal diagnosis	Unnecessary	Necessary
Treatment (dependent on severity)	Phototherapy (common)	Exchange transfusion (common newborn treatment)
	Exchange transfusion (rare)	Intrauterine transfusion (common antenatal treatment)
Types of antibody	IgG	IgG
Prophylaxis	None	RhIG
		Antenatal RhIG

TABLE 14-7 Frequency of Types of Autoimmune Hemolytic Anemia

Type of Autoimmune Hemolytic Anemia	Frequency
Warm AIHA	70%
Cold agglutinin syndrome	16%
Paroxysmal cold hemoglobinuria	1%–2%
Drug-induced	12%

frequency of disease in women than in men.¹⁶ The majority of individuals who develop WAIHA are older than 40 years of age.⁹

WAIHA may be idiopathic, with no underlying disease process (50% to 70% of cases), or it may be secondary to another disease process (30% to 50% of cases),¹⁷ such as lymphoid neoplasm, other neoplastic disorder, autoimmune or chronic inflammatory disorder, and viral infection. The disorders reported to be associated with WAIHA are listed in Box 14-3. Development of WAIHA may precede the diagnosis of one of these disorders. In one series of patients with idiopathic WAIHA, 18% were diagnosed with a lymphoid neoplasm during 2 years of follow-up.¹⁸

Signs and symptoms usually do not appear until significant anemia has developed. Pallor, weakness, dizziness, dyspnea, jaundice, and unexplained fever are occasionally presenting complaints. Hemolysis is usually extravascular and occurs predominantly in the spleen. The degree of anemia can be severe (hemoglobin less than 7.0 g/dL) or mild. The onset of

BOX 14-3 Disorders Associated With Warm Autoimmune Hemolytic Anemia

- Lymphoid neoplasms such as chronic lymphocytic leukemia, Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma, and Waldenström's macroglobulinemia
- Autoimmune disorders such as systemic lupus erythematosus, rheumatoid arthritis, scleroderma, and pernicious anemia
- Other neoplastic disorders, including carcinomas of the ovary, breast, lung, colon, pancreas, thymus, kidney, and uterus
- Viral infections, including hepatitis B and hepatitis A
- Chronic inflammatory disorders including ulcerative colitis

WAIHA is usually gradual and may be precipitated by a variety of factors, such as infection, especially viral infection,¹⁹ or after blood transfusion or organ transplantation.²⁰ WAIHA may predispose patients to thromboembolic disease.^{21,22}

Serological Evaluation

A positive DAT is present with a polyspecific antiglobulin reagent. On further analysis with monospecific antiglobulin reagents, the DAT result is frequently positive for both IgG and C3d (67% of cases). The remaining cases are positive for IgG (20%) or C3d (13%) alone.⁴

The serum of a patient with WAIHA usually demonstrates evidence of free autoantibody at low titer (e.g., weak reactivity). In 80% of the cases, the immunoglobulin is IgG alone or IgG together with IgA, IgM, and/or C3d.⁴ Complement proteins act synergistically with immunoglobulins to cause red cell hemolysis. In fact, the severity of hemolysis is greater

the presence of complement in addition to IgG.²³ Although not evaluated as a part of routine diagnostic testing, the presence of IgA, IgM, or both may be found in addition to IgG if appropriate antisera are used.²⁴ WAIHA can present several difficult problems in serological testing, which fall into two categories:

1. The patient's red cells are strongly coated with autoantibody, which interferes with phenotyping.
2. Autoantibody present in the serum may mask an underlying alloantibody.

Autoantibody Specificity

The autoantibodies produced in WAIHA usually react with all cells tested. Serological studies have suggested that some autoantibodies are directed at Rh blood group antigens because of their lack of reactivity with Rh_{null} cells (cells which lack all Rh blood group antigens).¹⁵ However, further analysis of these autoantibodies with apparent Rh specificity has demonstrated that the reactivity is actually directed at another red cell membrane protein that is also lacking in Rh_{null} cells.²⁰ On rare occasions, other specificities have also been reported.^{4,15,25}

DAT-Negative AIHA

Occasionally (in 1% to 3% of patients), the DAT result is repeatedly negative in a patient who has clear evidence of hemolysis with no other apparent cause.^{9,25} These patients represent a small group that is referred to as having DAT-negative AIHA.²⁵ More sensitive techniques for the detection of IgG, C3d, or both on red cells have shown that many of these patients have increased levels of these immunoproteins on their cells. The routine DAT can detect immunoglobulin sensitization of as little as 200 molecules of IgG per red cell.²⁵ More sensitive techniques are capable of detecting as few as 20 IgG molecules.²⁶ When interpreting DAT results, a positive DAT alone is not indicative of immune hemolysis, but if hemolysis is present or suspected, it could be the result of immune mechanisms. The DAT result can be positive in up to 8% of hospitalized patients who have no signs or symptoms of hemolysis.²⁵ In most of these patients, the positive result reflects complement sensitization, probably secondary to the disease process from which the patient is suffering.

IgA antibodies have also been reported to cause AIHA with characteristics of WAIHA or, less commonly, cold agglutinin disease.²⁷ These would also result in a negative DAT result since IgA does not fix complement *in vivo*.²⁸

Laboratory Diagnosis

Typically, patients with WAIHA exhibit a moderate to severe normocytic anemia with increased reticulocyte count. The blood smear can display classic signs of extravascular hemolysis: polychromasia reflecting reticulocytosis (see Fig. 14-4) and spherocytosis. Occasionally, nucleated red blood cells may be seen. On rare occasions, WAIHA is associated with reticulocytopenia (decreased reticulocyte count). Reticulocytopenia associated with intense hemolysis indicates a lack of bone marrow response and is associated with a high mortality rate. Patients also show accumulation of the products of red cell catabolism: hyperbilirubinemia (especially unconjugated) and increased LD.

Treatment

Therapy in WAIHA is aimed at treating the underlying disease if one is present. Measures to support cardiovascular function are important in patients who are severely anemic. Transfusion is usually avoided, if possible, as this may only accelerate the hemolysis instead of ameliorating the anemia. However, transfusion should be used in life-threatening situations.

As all donor blood is invariably incompatible, it is general practice to use donor blood that is least reactive in the crossmatch and antigen-negative for any clinically significant alloantibodies present in the patient's serum.^{29,30} Blood is transfused slowly, in small volumes (100 mL), and the patient observed closely for any adverse reactions.³¹ Some hematologists advocate the use of phenotypically similar blood irrespective of its degree of incompatibility in the crossmatch. The rationale for this approach is that patients with autoimmune antibodies may be more likely to produce alloimmune antibodies, which can be masked by the autoantibodies. However, one study indicates that the incidence of alloimmunization or adverse hemolytic transfusion reactions in patients with WAIHA is no greater than the incidence found in other multitransfused patient populations.³²

Corticosteroids are usually the first line of treatment. Corticosteroids such as prednisone produce their effect by:

- Reduction of antibody synthesis²⁵
- Altered antibody avidity³³
- Depression of macrophage activity,³³ which reduces the clearance of antibody-coated red cells²⁵

Splenectomy is usually considered as the next step if corticosteroid therapy is ineffective. Splenectomy decreases the production of antibody and removes the primary site of red cell destruction.²⁵ Immunosuppressive drugs, intravenous immunoglobulin, antilymphocyte globulin, anti-CD20 (Rituximab),³⁴ or plasma exchange may be used in patients who do not respond to conventional therapy. The success rate of these alternative therapies is variable, and they are used only in selected cases.^{17,23,35}

Cold Autoagglutinins

Normal Cold Autoagglutinins

Cold reacting autoantibodies (autoagglutinins) are present in all normal human sera.³⁶ The specificity of these cold autoantibodies includes anti-I, anti-H, and anti-IH. Practically all adults have I and H antigens present on their red cells. Generally, most examples of anti-I, anti-H, and anti-IH have no clinical significance, and these autoantibodies are often too weak to be detected via routine serological testing, owing primarily to their low concentration in the serum and their narrow thermal range (4°C to 22°C).³⁶ The characteristics of normal cold autoantibodies found in healthy adults with those of pathological cold autoantibodies are compared in Table 14-8. The benign autoagglutinins differ in many ways from the pathological cold autoagglutinins that produce cold agglutinin syndrome (or cold AIHA). The fundamental characteristic that differentiates benign autoagglutinins from pathological autoagglutinins is the thermal amplitude: pathological cold autoagglutinins may react at or above 30°C.³⁶

TABLE 14-8 Comparison of Characteristics of Normal and Pathological Cold Autoantibody

Characteristic	Normal	Pathological
Thermal amplitude	< 22°C	Broad: up to 32°C
Spontaneous autoagglutination	None	Significant degree that disperses on warming to 37°C
Titer	< 1:64 at 4°C	> 1:1,000 at 4°C
Albumin enhancement	None	Reactivity enhanced
Clonality of antibody	Polyclonal	Idiopathic = monoclonal Secondary to infection = polyclonal
Clinical significance	None	Causes cold AIHA
Usual antibody specificity	Anti-I	Anti-I
Direct antiglobulin test (DAT)	Negative or weak positive with polyspecific anti-globulin reagent	2 to 3+ with polyspecific antiglobulin reagent

Pathological Cold Autoantibodies

Pathological cold autoantibodies can be divided into three types:

1. Primary (idiopathic) cold agglutinin disease (primary CAD)
2. Cold agglutinin syndrome (CAS) secondary to infection
3. Paroxysmal cold hemoglobinuria (PCH)

Cold Agglutinin Disease (CAD)

Primary CAD Primary cold agglutinin, also called cold hemagglutinin disease or idiopathic cold AIHA, represents approximately 16% of the cases of AIHA.²⁵ Primary CAD is a chronic condition and occurs predominantly in older individuals, with a peak incidence after 50 years of age.²⁵ It is found in all racial groups, affecting both men and women. Although the disease is often idiopathic, a careful evaluation of the patient may reveal the presence of a lymphoproliferative disorder,¹⁵ other malignancy, or infection. Because of this association, it is prudent to investigate patients for possible malignancy when they present with a pathological cold autoantibody and no other obvious cause, such as infection. This is illustrated by one series of 78 patients with persistent cold agglutinin disease. On investigation, 28 of these patients were found to have no underlying malignancy, 6 had chronic lymphocytic leukemia, 31 had non-Hodgkin's lymphoma, and 13 had Waldenström's macroglobulinemia.³⁷

CAD is a hemolytic anemia produced by an autoantibody that reacts optimally at 4°C but has a wide thermal amplitude, reacting at temperatures greater than 30°C as well.^{25,36,38} The antibody is usually an IgM immunoglobulin, which quite

efficiently activates complement.³⁶ Antibody specificity in this disorder is almost always anti-I,³⁶ less commonly anti-i, and rarely anti-Pr.³⁶

CAD is rarely severe and is usually seasonal, as the cold winter months often precipitate the signs and symptoms of a chronic hemolytic anemia. **Acrocyanosis**, also called Raynaud's phenomenon³⁶ (symptoms of cold intolerance, such as pain and a bluish tinge in the fingertips and toes, owing to vasospasm), is frequently the patient's main complaint, along with a sense of numbness in the extremities when exposed to the cold. These symptoms occur because the cold autoantibody agglutinates the individual's red cells in the capillaries of the skin, causing local blood stasis.³⁶ During cold weather, the temperature of an individual's skin and exposed extremities can fall to as low as 28°C, activating the cold autoantibody. This activated cold antibody agglutinates red cells and fixes complement as the erythrocytes flow through the capillaries of the skin. When the erythrocytes return to the body core (where the temperature is 37°C), the cold agglutinin elutes off the red cells, leaving activated complement behind. Hemolysis occurs from the completion of the complement cascade or by removal of red cells sensitized with C3b/iC3b by macrophages in the liver (see the earlier section on Intravascular Hemolysis for a review). If any red cells coated with C3b/iC3b escape destruction in the liver, their complement proteins are further degraded to C3d, for which there are no receptors on macrophages.⁷ The patient's DAT result will be positive with monospecific anti-C3d antiglobulin reagents.

This hemolytic episode is not associated with fever, chills, or acute renal insufficiency, as would be characteristic of patients with paroxysmal cold hemoglobinuria (see later discussion). Hemoglobinemia and, less frequently, hemoglobinuria may be detected after exposure to the cold. Patients also display weakness, pallor, and weight loss, which are characteristic symptoms of chronic anemia. CAD usually remains quite stable, and when it does progress, it intensifies gradually. Other clinical features of CAD may include jaundice and splenomegaly.

Laboratory Findings Most patients with CAD present with reticulocytosis and a positive DAT result (polyspecific and C3d). In some cases, grossly visible agglutination of anticoagulated whole blood samples occurs as the blood cools to room temperature. As a result of this autoagglutination, performance of blood counts and preparation of blood smears may be difficult. The patient's mean corpuscular volume (MCV) from an automated cell counter will be erroneously high and the red blood cell count erroneously low due to clumping of red blood cells. This causes unrealistic values in other calculated parameters, such as hematocrit, mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). The blood sample should be warmed to 37°C for 30 to 60 minutes and reassayed on the automated cell counter to obtain accurate values. In severe cases, replacement of plasma in the prewarmed blood specimen with an equal volume of warm saline may be necessary to obtain accurate CBC results. The tendency for spontaneous autoagglutination of red cells from these patients dictates that serum samples must be

maintained and separated at 37°C to obtain accurate results for the antibody titer and thermal amplitude studies.³⁹ Similarly, samples for the determination of DAT results must be collected into ethylene diaminetetraacetic acid (EDTA) to inhibit any in vitro attachment of complement to the cells after collection.

A simple serum screening procedure can be performed by testing the ability of the patient's serum to agglutinate normal saline-suspended red cells at 20°C and 4°C. If this test result is positive, further steps must be taken to determine the titer and thermal amplitude of the cold autoantibody; if negative, the diagnosis of CAD is unlikely.³⁶

The peripheral blood smear in patients with CAD may show agglutination (clumping of red cells) (Fig. 14-5). The clinical criteria for diagnosis of CAD are summarized in Box 14-4.

Cold Agglutinin Syndrome (CAS) Cold agglutinin syndrome occurs as a transient disorder secondary to infections. Episodes of cold autoimmune hemolytic anemia often occur after upper respiratory infections. Approximately 50% of patients suffering from pneumonia caused by *Mycoplasma pneumoniae* have elevated titers (greater than 1:64) of cold autoagglutinins.^{25,36,38} Secondary CAS develops in the second or third week of the patient's illness, and a rapid onset of hemolysis with symptoms of pallor and jaundice is usually found. Resolution of the episode usually occurs in 2 to 3 weeks, as the hemolysis is self-limited.³⁶ The offending cold autoantibody is an IgM immunoglobulin with characteristic anti-I specificity. Very high titers of the cold autoagglutinin are seen almost exclusively in patients with mycoplasma pneumonia. The cold agglutinin produced in this infection is due to an immunological response to the mycoplasma antigens, and these antibodies cross-react with the red cell I antigen.^{40,41}

The antibodies produced in both primary CAD and CAS secondary to mycoplasma pneumonia have anti-I specificity. The autoantibody in primary CAD is invariably monoclonal (IgM with kappa [κ] light chains only), whereas the autoantibody produced secondary to infection in CAS is polyclonal (IgM with both κ and lambda [λ] light chain types).³⁶ The

BOX 14-4 Clinical Criteria for the Diagnosis of Cold Agglutinin Disease

- Clinical signs of an acquired hemolytic anemia, sometimes with a history of acrocyanosis and hemoglobinuria upon exposure to cold
- A positive DAT result using polyspecific antisera
- A positive DAT result using monospecific C3 antisera
- A negative DAT result using monospecific IgG antisera
- The presence of reactivity in the patient's serum owing to a cold autoantibody
- A cold agglutinin titer of 1:1,000 or greater in saline at 4°C with visible autoagglutination of anticoagulated blood at room temperature

monoclonality of the autoantibody in primary CAD suggests a possible underlying lymphoproliferative disorder.

Infectious Mononucleosis Infectious mononucleosis may also be associated with a hemolytic anemia resulting from a cold autoagglutinin. Many studies have reported an association of anti-i production in infectious mononucleosis. The percentage of patients with infectious mononucleosis who develop anti-i varies from 8% to 68%.^{34,39} The antibody is usually a low-titer IgM cold agglutinin with a narrow thermal range. A small number of these patients who develop anti-i produce a high-titer, IgM cold agglutinin with a wide thermal range,³⁹ which causes in vivo hemolysis. Acute illness with sore throat and high fever, followed by weakness, anemia, and jaundice, are characteristic features of infectious mononucleosis. For a review of infectious mononucleosis, see Chapter 16. The cold autoantibody specificity most commonly found in the various infections causing secondary CAD are outlined in Table 14-9.

Treatment Treatment of primary CAD, secondary CAD, and the anemia associated with infectious mononucleosis is similar. In many cases, the disease is self-limited and requires no treatment. Patients with persistent disease may be instructed to avoid the cold, keep warm, or move to a milder climate.³⁹ Additionally, patients may even be advised to avoid cold exposure when accessing the refrigerator and freezer and when consuming cold food and beverages. Corticosteroids have been used but have limited success.³⁵ Plasma exchange has been used in acute cases to provide temporary removal of antibodies.^{23,35} Patients with severe disease unresponsive to conventional therapy have been successfully treated with



FIGURE 14-5 Cold hemagglutinin disease (peripheral blood). Note the autoagglutination of red cells.

TABLE 14-9 Secondary Cold Autoimmune Hemolytic Anemia

Type of Infection	Cold Autoantibody Specificity
Mycoplasma pneumonia	Anti-I
Infectious mononucleosis	Anti-i
Lymphoproliferative disorder	Anti-I, i, or Pr

anti-CD20 (Rituximab).³⁹ Splenectomy is ineffective because extravascular hemolysis resulting from complement sensitization occurs predominantly in the liver.

Transfusion is rarely required. If blood is needed, the blood should be ABO/Rh compatible and lack any antigens for which the patient has an alloantibody. Blood should be warmed using a blood warmer and transfused slowly, with constant monitoring of the patient for adverse reactions.³⁶

Paroxysmal Cold Hemoglobinuria

Paroxysmal cold hemoglobinuria (PCH) is the least common type of AIHA, representing only 1% to 7% of patients.²⁵ It occurs most commonly in children, associated with viral disorders such as measles, mumps, chickenpox, infectious mononucleosis, and the poorly defined "flu syndrome."⁴² Although PCH is transient and self-limited, severe hemolysis may occur.

Originally, PCH was described in association with syphilis, in which an autoantibody was formed in response to *Treponema pallidum* organism, the causative agent of the disease.⁴³ However, with the discovery and use of antibiotics, PCH is no longer commonly associated with syphilis.

Red cell destruction in PCH is the result of a cold-reacting IgG autoantibody (always polyclonal) termed an **autohemolysin**. This autohemolysin most often binds to the Pantigen on the patient's red cells at lower temperatures. Hemolysis occurs when the red cells are rewarmed on return to the normal body temperature. The autohemolysin fixes complement, and the sensitized cells undergo complement-mediated intravascular hemolysis.⁴² The PCH autoagglutinin attaches only to red cells at cooler temperatures and then activates complement in warmer temperatures. Thus, the antibody is called a biphasic hemolysin. It is also called the Donath–Landsteiner antibody, and its specificity is usually anti-P.³⁶

As the name of PCH implies, paroxysmal or intermittent episodes of hemoglobinuria occur on exposure to the cold. These acute attacks may be characterized by a sudden onset

of fever, shaking chills, malaise, abdominal cramps, and back pains.⁴² All the signs of intravascular hemolysis are evident, including hemoglobinemia, hemoglobinuria, and hyperbilirubinemia (see Fig. 14–3). This results in a severe and rapidly progressive anemia. Polychromasia, nucleated red blood cells, and poikilocytosis are demonstrated in the peripheral blood smear. The symptoms and signs may resolve in a few hours or persist for days. Splenomegaly and renal insufficiency may also develop. PCH and cold agglutinin syndrome are compared and contrasted in Table 14–10.

The **Donath–Landsteiner test** is the classic diagnostic test for PCH, developed by the two physicians for whom it is named. A blood sample drawn from the patient is split into two aliquots maintained at different temperatures. One aliquot, used as the control, is kept at 37°C for 60 minutes. The other aliquot is cooled at 4°C for 30 minutes and then incubated at 37°C for another 30 minutes. Both samples are then centrifuged and observed for hemolysis. A positive test result is present when hemolysis is seen in the sample placed at 4°C and then at 37°C, and no hemolysis in the control sample. The Donath–Landsteiner test is summarized in Table 14–11.

Treatment For acute postinfection forms of PCH, the hemolysis usually terminates spontaneously after resolution of the infectious process. Protection from cold exposure is usually the only useful therapy for PCH. However, a 4-year-old boy was recently successfully treated for PCH with a single dose of eculizumab, an anticomplement antibody.¹⁶ If anemia is severe, transfusions may be required. The same transfusion protocol as in CAD applies. Characteristics of warm and cold autoimmune hemolytic anemias are reviewed and compared in Table 14–12.

Mixed Autoimmune Hemolytic Anemia In the past two decades, a number of reports have drawn attention to the occurrence of mixed AIHA, in which patients exhibit autoantibodies

TABLE 14-10 Comparison of Paroxysmal Cold Hemoglobinuria and Cold Agglutinin Syndrome

Factor	PCH	Cold Agglutinin Syndrome
Patient population	Children or young adults	Elderly or middle-aged
Pathogenesis	Following viral infection	Idiopathic, lymphoproliferative disorder or following <i>Mycoplasma pneumoniae</i> infection
Clinical features	Hemoglobinuria: acute attacks upon exposure to cold (symptoms resolve in hours or days)	Acrocyanosis, autoagglutination of blood at room temperature
Severity of hemolysis	Acute and rapid	Chronic and rarely severe
Hemolysis	Intravascular	Extravascular or intravascular
Autoantibody	IgG (usually anti-P specificity, biphasic hemolysin)	IgM (anti-I/I, monophasic)
DAT	3+ (polyspecific)/neg IgG/3–4+ C3 monospecific	3+ (polyspecific)/neg IgG/3–4+ C3 monospecific
Thermal range	Moderate (< 20°C)	High (up to 30–31°C)
Titer (4°C)	Moderate (< 1:64)	High (> 1:1,000)
Donath–Landsteiner test	Positive	Negative
Treatment	Supportive (disorder terminates when underlying illness resolves)	Avoid the cold

TABLE 14-11 Donath-Landsteiner Test

	Whole Blood Control	Whole Blood Test
Procedure		
1. 30 min	37°C	4°C
2. 30 min	37°C	37°C
3. Centrifuge and observe		
Results		
Positive	No hemolysis	Hemolysis
Negative	No hemolysis	No hemolysis
Inconclusive	Hemolysis	Hemolysis

having the characteristics of both warm and cold autoantibodies.⁴⁴⁻⁴⁶ Less than 10% of cases of AIHA are considered mixed.¹⁶ Patients with mixed-type AIHA usually present with a severe, acute condition.⁴⁷ They may exhibit signs of extravascular hemolysis from IgG antibodies and intravascular hemolysis from IgM or complement activation. Both warm and cold autoantibodies may be present because a number of the lymphoproliferative and collagen diseases may be associated with either form of autoantibody.^{4,46} Approximately half of the cases of mixed AIHA are idiopathic and the remainder are associated with autoimmune diseases such as systemic lupus erythematosus.^{23,35}

Drug-Induced Immune Hemolytic Anemia

The administration of drugs may lead to the development of a wide variety of hematologic abnormalities, including immune hemolytic anemia. Drug-induced immune hemolytic anemia represents approximately 12% of cases of immune hemolytic anemia in various studies.²⁵ Historically, three mechanisms have been described that lead to the development of drug-induced immune hemolytic anemia, and a fourth mechanism leads to the development of a positive DAT but is not associated with hemolysis. Sufficient new data have emerged to perhaps reclassify these mechanisms. Nevertheless, it is instructive to review the traditional mechanisms.

Autoimmune Mechanism The autoimmune mechanism is the most common drug-induced immune hemolytic anemia, accounting for approximately 70% of all cases.²⁵ The antibodies produced by this mechanism are considered “true autoantibodies,” because they react against intrinsic red blood cell antigens, not the drug or the drug-erythrocyte complex. The drugs implicated in this response include the antihypertensive drug α -methyldopa (Aldomet) and related drugs (L-dopa, procainamide)⁴⁸ (Box 14-5). Drug-induced AIHA by this mechanism is difficult to diagnose because it mimics WAIHA. It has been suggested the autoantibodies produced in response to these drugs are the result of altered red cell antigens not recognized as self; however, the exact mechanism is still unknown.⁴⁹ A positive DAT develops in approximately 12% to 15% of the patients receiving α -methyldopa, and 1% to 3% of these patients go on to develop AIHA. The antibodies produced by patients suffering from this disorder react weakly with all cells tested or demonstrate specificities similar to those found in WAIHA. Hemolysis is extravascular and the DAT result is strongly positive with anti-IgG and negative with anti-C3. Patients may continue to have a positive DAT result for up to 2 years after discontinuation of the drug.

Drug Adsorption (hapten) Mechanism The drug adsorption (hapten) mechanism is the second most common mechanism of drug-induced hemolytic anemia. The drugs implicated in this response include the penicillins and the cephalosporins.²⁵ This mechanism requires two components (Fig. 14-6). First, the drug is nonspecifically adsorbed to the patient's red cells and remains firmly attached. Second, once adsorbed, the drug must be able to elicit an antibody response. The drug antibody is usually IgG and reacts only with drug-treated red cells. Large doses of intravenous penicillin (10 million units daily) are needed to produce an immune response.²⁵ Approximately 3% of patients on high-dose intravenous penicillin develop drug antibodies causing a positive DAT, but only 5% of these patients have actual clinical hemolysis.²⁵

Laboratory findings include signs of extravascular hemolysis. The disorder develops over a period of 7 to 10 days. The DAT results are strongly positive with anti-IgG and negative with anti-C3.

TABLE 14-12 Comparison of Autoimmune Hemolytic Anemias

Factor	Warm Autoimmune Hemolytic Anemia (WAIHA)	Cold Agglutinin Disease (CAD)	Paroxysmal Cold Hemoglobinuria (PCH)
Autoantibody optimal reactivity temperature	> 37°C	< 4°C (can react at temperatures greater than 30°C)	< 4°C
Immunoglobulin class	IgG (Rarely IgA or IgM)	IgM	IgG
Complement activation	May bind complement	Binds complement	Binds complement
Hemolysis	Usually extravascular	Primarily extravascular	Intravascular
Frequency	70%–75% of cases	16% of cases	1%–2%
Specificity	Frequently Rh	Mostly anti-I, less commonly anti-i, and rarely anti-Pr	Usually anti-P

BOX 14-5 Partial List of Drugs Associated With Positive DAT or Hemolytic Anemia by Mechanism

Methylidopa-Induced (Autoimmune) Mechanism

- Methylidopa
- Ceftriaxone
- Chlorpromazine
- Ibuprofen
- Levodopa
- Mefenamic acid
- Nomifensine
- Procainamide
- Thioridazine

Drug Adsorption Mechanism

- Cephalosporins
- Diclofenac
- Penicillins

Immune Complex Mechanism

- Acetaminophen
- Antihistamines
- Cephalosporins
- Chlorpromazine
- Diclofenac
- Isoniazid
- Quinidine
- Quinine
- Rifampin
- Streptomycin
- Sulfonamides
- Stibophen
- Tetracycline

Membrane Modification Mechanism (Protein Adsorption)

- Cephalosporins

Note: Some drugs may act by more than one mechanism.

Immune Complex Mechanism The immune complex mechanism is the least common drug mechanism in drug-induced hemolytic anemia. The most common drug involved in this response is quinidine.²⁵ Other drugs associated with the immune complex mechanism are listed in Box 14-5. The patient responds to these drugs by producing an antibody (IgG, IgM, or both) against the drug that binds to the drug, forming an antibody-drug immune complex (Fig. 14-7). The antibody-drug complex then adsorbs onto the patient's red cells, and complement is activated. The antibody-drug immune complex is merely adsorbed onto the red cell membrane (not bound to it) and easily disassociates, leaving activated complement behind. Only a small amount of the drug is necessary to produce this response.

Laboratory findings include evidence of intravascular hemolysis with hemoglobinemia and hemoglobinuria. The DAT result is positive with complement components only because the immune complex has disassociated. In vitro agglutination reactions are generally observed during serological testing only when the drug is added to the patient's serum and test red cell mixture.

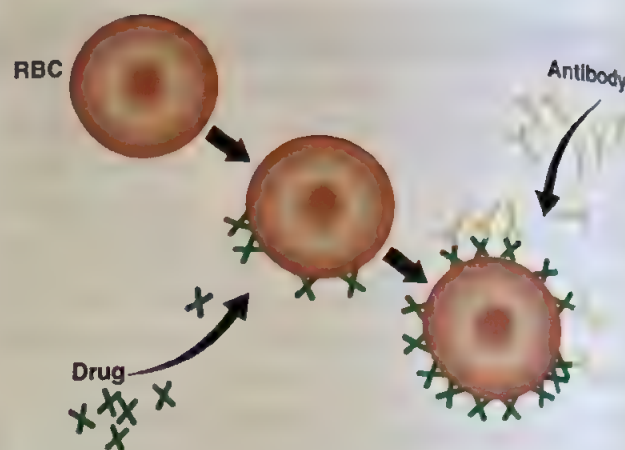


FIGURE 14-6 Drug adsorption mechanism. (From Petz LD, Garratty G, editors. *Acquired Immune Hemolytic Anemias*. New York: Churchill Livingstone; 1980, with permission.)

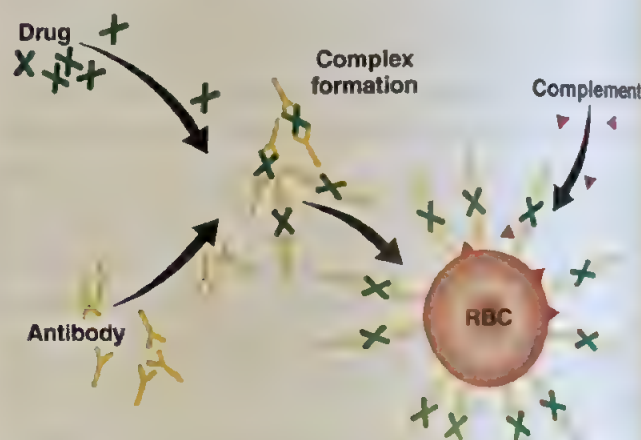


FIGURE 14-7 Immune complex mechanism. (From Petz LD, Garratty G, editors. *Acquired Immune Hemolytic Anemias*. New York: Churchill Livingstone; 1980, with permission.)

The primary treatment is to stop the drug if the patient is anemic because of active hemolysis. Corticosteroids may also be given. Interestingly, some of the drugs producing hemolysis by this mechanism are also associated with the development of drug-induced immune thrombocytopenia (ITP). The antidrug immune complex also adsorbs onto the platelets.⁴⁹ However, it is rare to find a patient with simultaneous hemolysis and thrombocytopenia caused by antibodies to a single drug.

Membrane Modification Mechanism (Protein Adsorption) As the name implies, the drug modifies the red cell membrane so that normal plasma proteins are nonspecifically adsorbed onto the patient's red cells (Fig. 14-8). Cephalosporins are also the drugs most commonly implicated in this response.²¹ The red cells become coated with numerous plasma proteins such as albumin, fibrinogen, and globulins. Approximately 3% of patients receiving the drug develop a positive DAT result owing to the nonspecific immunoglobulin adsorption by the red cells. Hemolytic anemia has not been reported in association with this mechanism of drug-induced positive DAT.

The preceding classification used for the drug-induced immune hemolytic anemias provides a convenient mechanistic approach to how drugs may be implicated in immune hemolysis. Recent reports have demonstrated immune hemolytic

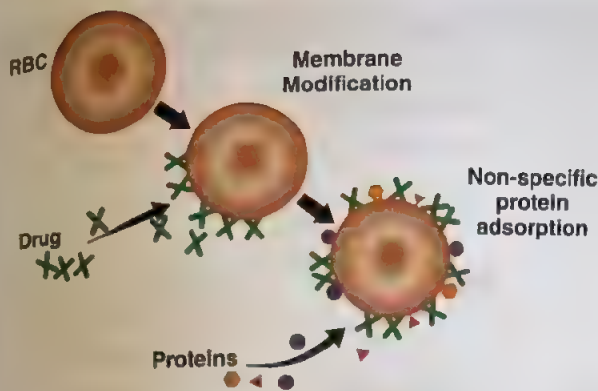


FIGURE 14-8 Membrane modification mechanism. (From Petz LD, Garratty G, editors. *Acquired Immune Hemolytic Anemias*. New York: Churchill Livingstone; 1980, with permission.)

anemia from certain drugs with more than one mechanism. More recently it has been suggested that only a single mechanism may be responsible for all drug-related immune hemolysis (unifying theory).⁴⁹ The four mechanisms of drug-related anemia are compared in Table 14-13. The antibody characteristics of the various types of autoimmune hemolytic anemias are contrasted in Table 14-14.

Nonimmune Hemolytic Anemia

Acquired nonimmune hemolytic anemias represent a diverse group of conditions associated with the shortened survival of red cells by various mechanisms. Often a number of mechanisms are operative at the same time; for example, malaria

leads to mechanical destruction of red cells, and, in addition, immunological factors play a role in shortened red cell survival. Classifications may be made along either causative or mechanistic lines. A classification incorporating both approaches is provided in Table 14-15.

Intracellular Infections

Infections with parasites that invade red blood cells can cause cell hemolysis and subsequently anemia. Malaria and *Babesiosis* infections are included in this category. This section describes how these intracellular infectious agents can cause hemolytic anemia.

Malaria

Malaria is the most common protozoal infection in humans. It has a high incidence in the tropical and subtropical regions of the world, accounting for a fair percentage of the anemia in those regions. According to the World Health Organization (WHO), climate change is predicted to increase both the range and intensity of malaria transmission.⁵⁰ The mosquito's habitat is affected by the shift of tropical and subtropical regions. It has been estimated that more than 200 million people suffer from the disease worldwide, resulting in the deaths of more than 400,000 annually.⁵⁰ Most fatalities occur in nonimmune children; those who survive a childhood infection invariably suffer from an ongoing debilitating disease. Globally, malarial incidence rate declined between 2010 and 2018, from 71 to 57 cases per 1,000 population at risk.⁵⁰ However, the global fight against malaria continues to be hampered by increases in both drug and insecticide resistance.⁵⁰

TABLE 14-13 Mechanism Leading to Development of Drug-Related Antibodies

Mechanism	Prototype Drugs	Immunoglobulin Class	DAT	Biological Results	Frequency of Hemolysis
Immune complex formation (innocent bystander)	Quinidine	IgM or IgG	Positive (often to complement fragments only; however, IgG may be present)	Eluate often negative	Small doses of drug may cause acute intravascular hemolysis with hemoglobinemia and hemoglobinuria; renal failure is common
Drug adsorption	Penicillins	IgG	Positive (strongly) due to IgG sensitization	Eluate often negative	3%–4% of patients on large doses (10 million units) daily of penicillin, which is one of the most common causes of drug-induced immune hemolysis, usually extravascular in nature
Membrane modification (nonimmunologic protein adsorption)	Cephalosporins	Numerous plasma proteins (nonimmunologic sensitization)	Positive due to a variety of serum proteins	Eluate negative	No hemolysis; however, 3% of patients receiving the drug develop a positive DAT
Autoimmunity	Methyldopa (Aldomet)	IgG	Strongly positive (due to IgG sensitization)	Eluate positive (warm autoantibody identical to antibody found in WAIH)	0.8% develop a hemolytic anemia that mimics a WAIHA (depends on the dose of the drug); 15% of patients receiving Aldomet develop a positive DAT

TABLE 14-14 Antibody Characteristics in Autoimmune Hemolytic Anemia

Characteristic	Warm Reactive Autoantibody	Cold Reactive Autoantibody	Paroxysmal Cold Hemoglobinuria (PCH)	Drug-Related Autoantibody
Immunoglobulin characteristics	Polyclonal IgG; IgM, and IgA may also be present; rarely IgA alone	Polyclonal IgM in infection Monoclonal κ chain IgM in cold agglutinin disease	Polyclonal IgG	Polyclonal IgG
Complement activation	Variable	Always	Always	Depends on mechanism of drug, antibody, and RBC interaction
Thermal reactivity	20°C–37°C; optimum 37°C	4°C–32°C optimum 4°C; occasionally to 37°C	4°C–20°C; biphasic hemolysin	20°C–37°C; optimum 37°C
Titer of free antibody	Low (< 1:32) May only be detectable using enzyme treated cells	High (> 1:1,000 at 4°C)	Moderate to low (< 1:64)	Depends on mechanism of drug, antibody, and RBC interaction
Reactivity of eluate with antibody screening cells	Usually panreactive	Nonreactive	Nonreactive	Panreactive with Aldomet-type antibody Nonreactive in all other circumstances
Most common specificity	Anti-Rh precursor -common Rh –LW -En ^a /Wr ^b -U	-I -i -Pr	Usually anti-P	Anti-e-like; Aldomet, antidrug
Site of RBC destruction	Extravascular: predominantly spleen with some liver involvement	Extravascular: pre-dominantly liver, rarely intravascular	Intravascular	Intravascular and spleen

TABLE 14-15 Classification of Nonimmune Acquired Hemolytic Anemias

Classification	Examples	Mechanisms
Infections		
Intracellular	Malaria	Physical disruption and immune
	Babesiosis	Physical disruption
Extracellular	Bartonella	Direct action on RBC membrane and MPS sequestration
	Clostridium	Enzymatic action on RBC membrane
	Bacterial sepsis: meningococcal, pneumococcal	Physical disruption secondary to DIC
	Viral	Unknown
Mechanical		
Macroangiopathic	Cardiac prosthesis	Physical disruption because of shear stress
	March hemoglobinuria	Physical disruption
Microangiopathic	Hemolytic uremic syndrome (HUS)	Physical disruption
	Thrombotic thrombocytopenic purpura (TTP)	Physical disruption
Chemical and Physical Agents		
Oxidative agent	Dapsone at high dosage	Direct oxidation of RBC membrane components
Nonoxidative agents	Lead	Alteration of RBC membrane components
	Venoms	Possible direct effect on RBC membrane by enzymes

TABLE 14-15 Classification of Nonimmune Acquired Hemolytic Anemias—cont'd

Classification	Examples	Mechanisms
Osmotic effect	Water drowning or water ingestion during surgery	Osmotic lysis
	Burns	Localized dehydration
Acquired membrane disorders	Vitamin E deficiency, abetalipoproteinemia	RBC membrane oxidation; lack of membrane deformability
	Liver disease	Lipid abnormalities of RBC membrane lead to decrease in deformability
	Renal disease	Retained metabolic products cause membrane changes, leading to a decrease in deformability
Hypersensitivity		Sequestration of normal cells

DK = disseminated intravascular coagulation; MPS = mononuclear phagocyte system.

There are four species of malaria that can infect humans: *Plasmodium vivax*, *P. falciparum*, *P. ovale*, and *P. malariae*. *Plasmodium vivax* and *P. falciparum* are responsible for most infections producing disease in humans. In the case of *P. falciparum*, the disease can have a rapid and often fatal course. *Plasmodium malariae* and *P. ovale* infections are uncommon. *Plasmodium ovale* infections are confined to certain areas of Africa.

The number of patients presenting with malaria is also increasing in countries such as the United States, Europe, and Australia because of increased travel to endemic tropical and subtropical regions. It is estimated that more than 30,000 international travelers contract malaria each year.⁵¹

Life Cycle The malarial parasite has a complex life cycle. The insect vector is the *Anopheles* mosquito, of which numerous species can transmit the parasite. Figure 14-9 illustrates the malarial life cycle.

Clinical Presentation The most commonly reported symptoms are fever, malaise, headache, chills, and sweats. Some patients show classic periodic episodes of fever and chills, correlating with rupture of infected erythrocytes. Nausea, vomiting, and diarrhea may be present. Quite often, classic periodic episodes of fever and chills are absent, and these symptoms are mistakenly attributed to a viral infection, allowing the malaria to go untreated. It is always advisable to inquire if the patient has been overseas and which countries have been visited.

Splenomegaly is present in 40% to 50% of patients with acute malaria. It is present in virtually all patients with chronic malaria, accounting for the high incidence of splenomegaly in the tropics.⁵²

Laboratory Diagnosis Hemolysis of red cells occurs intravascularly as a result of direct red cell destruction by the parasite. In addition, malarial infection causes immune activation with increased monocyte/macrophage activity, which promotes extravascular hemolysis of both infected and noninfected red cells in the spleen.⁵³

Anemia associated with malaria is normocytic normochromic. Leukopenia is present in many cases, as is thrombocytopenia (particularly in individuals with *P. falciparum*

infections). Diagnosis of malaria is made by examination of a peripheral blood smear. Blood should be taken just before the onset of fever because the parasitemia is greatest at this time. However, this is possible only if classic periodic episodes of fever and chills are present. It is best to make blood smears from a fingerstick. If anticoagulated blood must be used, smears should be made as soon as possible to prevent changes in erythrocyte and parasite morphology.⁵⁴

Examination of an unfixed Giemsa- or Wright-stained blood smear (thick preparation) is performed to ascertain the presence of malarial parasites. Staining is usually performed at a pH of 7.2 to enhance the blue staining of the parasites' cytoplasm. Determination of the species of parasite may be made on this smear if schizonts are present (by the number of merozoites within the schizont). If only trophozoites are present, the examination of a thin blood smear is necessary. Often more than one parasite is present in a single cell (Fig. 14-10). *Plasmodium falciparum* gametocytes have a characteristic banana or crescent shape, assisting in the identification. Occasionally it is possible to see irregular purple inclusions called Maurer's dots in the red cell cytoplasm, which are probably breakdown products of hemoglobin. *Plasmodium vivax* gametocytes are round, ameboid forms that expand and distort the red cell. Bluish purple inclusions called Schüffner's dots are often seen in red cells infected with *P. vivax* and *P. ovale* (Fig. 14-11). The features of the different malarial parasites infecting humans are summarized in Table 14-16.

Immunoassays help screen large numbers of patients for the presence of infection. Flow cytometry has also been used to show the presence of malarial parasites in red cells.

Babesiosis

Infection by the organism *Babesia* represents a zoonotic infection, as humans are not natural hosts for the parasite. The disease is carried by ticks (*Ixodes scapularis*) and normally infects cattle, deer, and rodents.⁵⁵ The disease is usually tickborne in humans but has also been transmitted by blood transfusion.^{56,57} In the United States, cases are most common in New England and the upper Midwest, owing to the presence of infected ticks and their hosts in those areas.⁵⁷ The geography of the tick's habitat has rapidly increased over

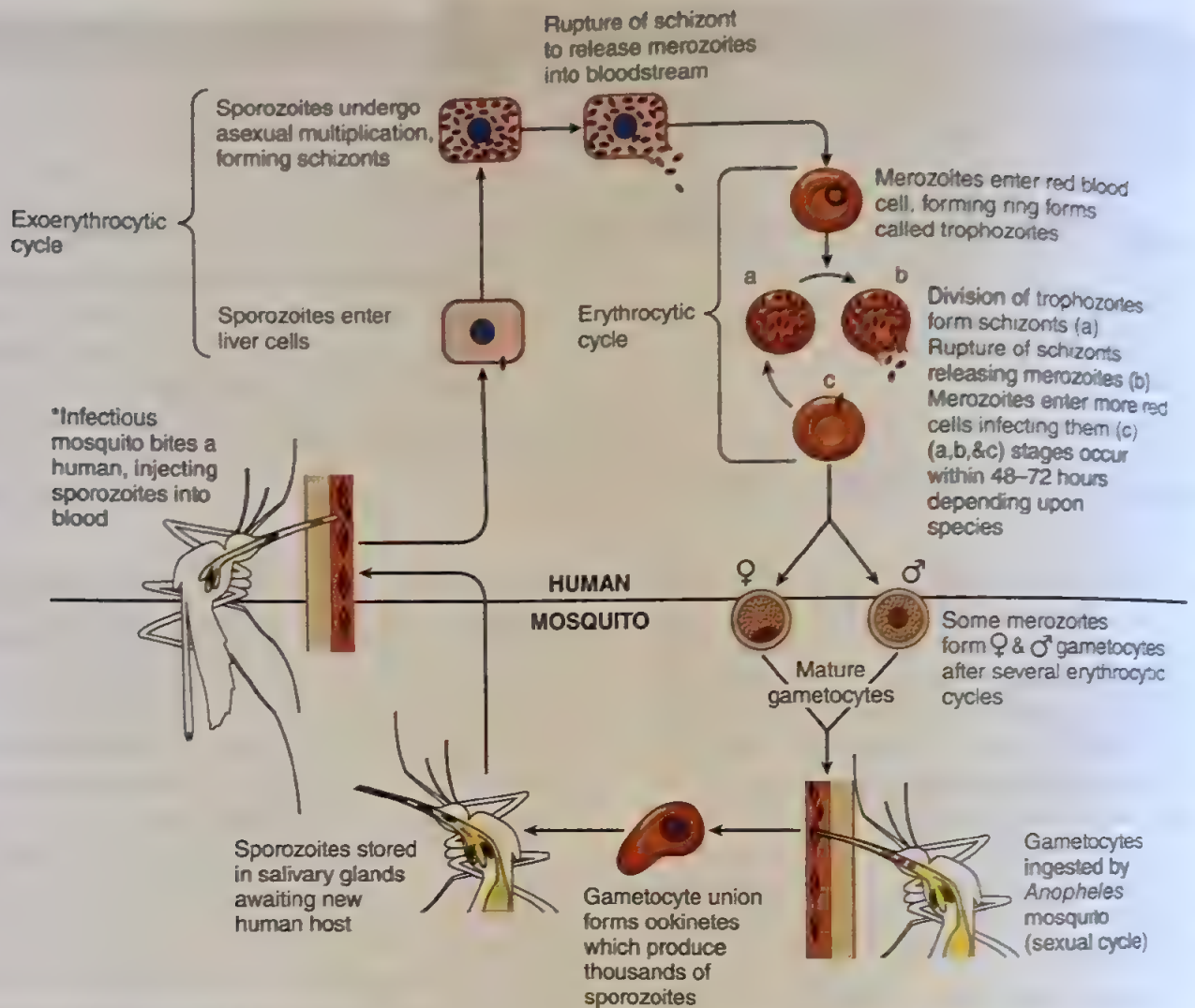


FIGURE 14-9 Malarial life cycle in humans and mosquitoes. Beginning of cycle is indicated by an asterisk.

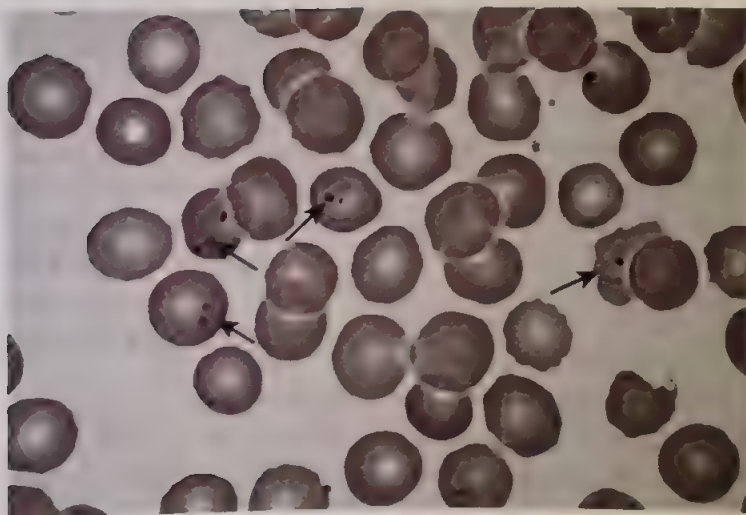


FIGURE 14-10 Ringed forms of *Plasmodium falciparum* in red blood cells (RBCs). Note the same RBCs may be infected with more than one ring.

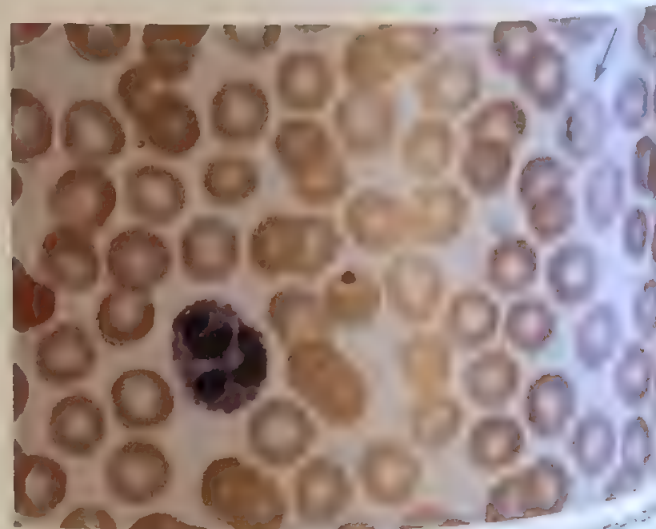


FIGURE 14-11 Late stages of *Plasmodium vivax* malaria. Note and contrast the platelet on the RBC (center) with the ring toward the periphery (arrow).

the past 20 years and is thought to be influenced by environmental changes as well as urbanization and agricultural practices.⁵⁶ Infection tends to be self-limited, although in elderly, immune-compromised, or asplenic patients, it can follow an acute and fatal course.⁵⁷ Patients usually present with a history of malaise, headache, and fever, sometimes associated with vomiting and diarrhea. In splenectomized patients, this

condition can progress to rigors, acute intravascular hemolysis with associated hemoglobinemia, hemoglobinuria, and renal failure.

Laboratory Diagnosis Diagnosis of the disease is made via examination of the peripheral blood, where parasites very similar to *P. falciparum* are seen in the

TABLE 14-16 Features of Malarial Parasites Infecting Humans

Feature	<i>P. falciparum</i>	<i>P. vivax</i>	<i>P. malariae</i>	<i>P. ovale</i>
Incubation period (days)	6–10	10–12	13–16	10–12
Asexual life cycle (h)	48	48	72	48
RBCs infected	All	Reticulocytes	Senescent	Reticulocytes
Secondary exoerythrocytic development	No	Yes	Yes	Yes
Duration of relapses in untreated patients	Not applicable	3–5 yr	Up to 40 yr	3–5 yr
Level of parasitemia	50%–60%	2%–5%	2%–3%	2%–3%
Ring form	Small, delicate, may have two chromatin dots, often on edge of RBC	Large, irregular, poor outline one chromatin dot	Large thick, prominent chromatin dot	Large irregular, poor outline
Schizonts	Maurer's dots Rarely seen in peripheral blood	Schüffner's dots Large, about same size as RBC	Schüffner's dots Small "daisy-head" 6–16 merozoites	Schüffner's dots Irregular arrangement 4–16 merozoites
Gametocytes	8–32 merozoites Crescent or sausage shape	12–25 merozoites Round and expanded RBC	Round, same size as RBC	

(Fig. 14-12). Babesiosis can be distinguished from malaria by the formation of tetrads of merozoites (Maltese cross), absence of pigment granules in infected erythrocytes, and the presence of extracellular merozoites.⁵⁵ A history of possible exposure to ticks and a lack of recent travel to areas where malaria is endemic help in making the correct diagnosis. Serological tests for antibodies to *Babesia* by immunofluorescent assay or testing by polymerase chain reaction (PCR) have been described.^{56,57}

Extracellular Infections

Other infectious agents that do not enter the red blood cell can still cause hemolysis. These are described in this section, along with differentiating features among laboratory findings and clinical features.

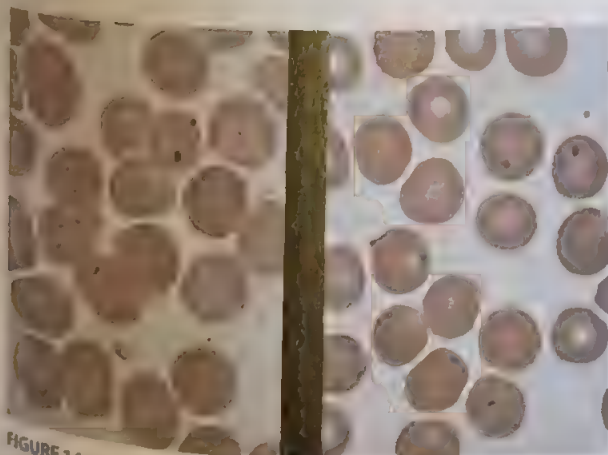


FIGURE 14-12 Comparison of babesiosis (left) and malaria (right).

Bartonellosis (Oroya Fever)

Bartonellosis is restricted to northern areas of South America, including Peru, Ecuador, and Columbia. The name "Oroya fever" derives from the city of Oroya in the Peruvian Andes, where many railroad construction workers were affected by the disease in the late 1800s. It is also referred to as Carrion's disease,⁵⁹ named after the medical student who died as a result of a self-experiment designed to determine the nature of the infection.

Bartonellosis has a high fatality rate in nonimmune patients and is caused by the organism *Bartonella bacilliformis*. Infection is transmitted by the sand fly (*Phlebotomus*), and there does not appear to be any intermediate host.

The disease has two clinical phases: The acute hemolytic phase, during which the organisms adhere to the red cell surface and appear as gram-negative rods, and the recovery phase, during which the organisms assume a coccoid appearance. The hemolytic phase (Oroya fever) may not occur in all patients. When it does occur, there is a rapid onset with marked intravascular hemolysis. Red cells are also sequestered in the spleen and liver.⁶⁰ The anemia can be quite severe, and blood smears show many nucleated red cells and a reticulocytosis.⁶⁰ Antibiotic therapy, including penicillin, streptomycin, and tetracyclines, is effective in treating patients in this stage of the infection.⁵² The second stage of the disease (verruca peruviana) is nonhematologic and involves the development of verrucous nodes (wartlike tumors) over the patient's face and extremities.

Clostridium Perfringens (WELCHII)

The *C. perfringens* organism is a gram-positive, spore-forming bacillus responsible for the development of gas gangrene

Infections with this organism are generally located in deep tissues where anaerobic conditions required for the organism's survival exist. The organism is normally present in the environment and may infect tissues exposed by trauma and surgical procedures. Hospital-acquired infections have been associated with gynecological procedures, Caesarean section, amniocentesis, cordocentesis, abortions, and molar pregnancies.⁶¹ The organism is responsible for extensive tissue damage resulting from the release of enzymes and toxins. Septicemia caused by *C. perfringens* may produce an acute intravascular hemolytic process resulting from the release of an alpha (α) toxin or lecithinase. This process, combined with phospholipases and possibly proteinases also produced by the organism, acts on the red cell membrane to cause its destruction and subsequent lysis of the cell.⁶² Hemolysis is often severe, with marked hemoglobinemia and hemoglobinuria. Acute renal failure may develop quite rapidly, and the prognosis is generally poor.^{63,64} Microspherocytes, hemolyzed "ghost cells," and left shift in neutrophils with toxic changes are common findings in the peripheral blood smear. Thrombocytopenia is present in most cases. Improvements in the maintenance of aseptic conditions during and following surgery have caused this form of hemolytic anemia to become quite uncommon.

Other organisms associated with hemolytic anemia are listed in Table 14-17.

CRITICAL THINKING QUESTION

14-3 The presence of which cellular morphology is a strong indicator of extravascular hemolysis? Why?

Mechanical Etiologies

The passage of red cells through the vascular system subjects the cell to a wide range of environmental conditions. As red

cells travel around the body, shear forces are highly variable and are influenced by:

1. The surface conditions of the blood vessel
2. The size of the vessel lumen
3. The rate at which the cell is moving
4. The number of other cells present at the same time
5. Other environmental conditions the cell is exposed to including changes in pH, electrolytes, and protein concentration

When these factors cause mechanical rupture of the cell membrane, intravascular hemolysis results, accompanied by the presence of red cell fragments or schistocytes on peripheral blood smear (Fig. 14-13).

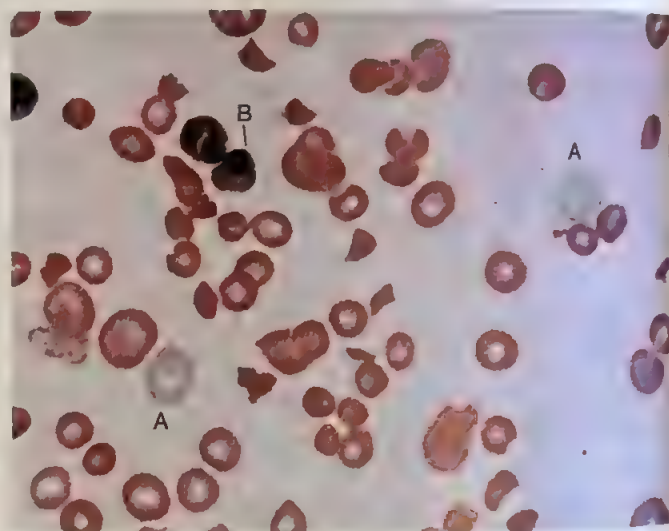


FIGURE 14-13 Peripheral blood showing red cell fragmentation with thrombocytopenia. (A) Polychromasia and (B) nucleated RBCs from a patient with thrombotic thrombocytopenia purpura (TTP).

TABLE 14-17 Organisms Associated With Hemolytic Anemia

Organism	Viruses	Protozoa	Fungi
<i>Bartonella bacilliformis</i>	Coxsackie	<i>Babesia microti</i>	<i>Aspergillus</i>
<i>Clostridium perfringens</i>	Cytomegalovirus	<i>B. divergens</i>	
<i>Escherichia coli</i>	Epstein-Barr	<i>Plasmodium falciparum</i>	
<i>Haemophilus influenzae</i>	Herpes simplex	<i>P. malariae</i>	
<i>Mycobacteria tuberculosis</i>	Influenza A	<i>P. ovale</i>	
<i>Mycoplasma pneumoniae</i>	Rubeola	<i>P. vivax</i>	
<i>Neisseria meningitidis</i>	Varicella	<i>Toxoplasma</i>	
<i>Salmonella</i> sp.			
<i>Shigella</i> sp.			
<i>Streptococcus</i> sp.			
<i>Vibrio cholera</i>			
<i>Yersinia enterocolitica</i>			

Cardiac Prosthesis

Historically, hemolytic anemia associated with prosthetic heart valves was a frequent complication of cardiac corrective surgery. Innovative changes in design and composition of valves have reduced mechanical hemolysis to a rare and minor complication.⁶⁵

The primary cause of hemolysis is mechanical trauma to red blood cells, resulting from turbulence of flow through the prosthesis.⁶⁶ The severity of the anemia is highly variable in patients with heart valve prostheses. Mild, compensated hemolysis is common; overt anemia is unusual, and rarely is the anemia severe enough to require transfusion.⁶⁵

The peripheral blood smear shows many fragmented cells (schistocytes), helmet cells, and occasional spherocytes (Fig. 14-14). The reticulocyte count and serum LD level are usually elevated.⁵⁰ Leukocytes are usually normal, and platelets are often reduced because of their interaction with the abnormal surface.⁶⁶ Decreased haptoglobin, mild hemoglobinemia, mild hemosiderinuria,⁶⁶ and occasionally hemoglobinuria are present (depending on the amount of red cell destruction).

Treatment of cardiac hemolysis can range from supportive therapy, which may include iron supplementation and transfusion, to surgically correcting the faulty valve or vessel.⁶⁷

March Hemoglobinuria

This form of hemolytic anemia was first described in the late 1800s in a young German soldier who demonstrated frank hemoglobinuria following a field marching exercise.⁶⁸ The anemia has been described in individuals involved in strenuous and sustained physical activity.⁶⁸ Similar traumatic red cell destruction has been reported in a practitioner of karate and a conga drum player.⁶⁸ The cause of the anemia is complex, involving direct physical disruption of red cells as they flow through the capillaries of the feet or hands, iron loss in sweat, and adaptation to a right-shifted oxygen dissociation curve.⁶⁹

Patients with march hemoglobinuria usually demonstrate a normal hemoglobin, although there may be an increase in the reticulocyte count. Hemoglobinemia and hemoglobinuria are episodic and present only after exercise. This obvious

association with exercise is helpful in distinguishing the hemoglobinuria from other causes such as paroxysmal nocturnal hemoglobinuria (see Chapter 13). Fragmented red cells are not a feature of this condition.

Treatment involves wearing cushion-soled shoes or running on softer surfaces.

Microangiopathic Hemolytic Anemia

Microangiopathic hemolytic anemia (MAHA) refers to a group of clinical disorders characterized by fragmentation of the red cells as they pass through abnormal arterioles, resulting in intravascular hemolysis.⁷¹ Most often the abnormalities in the microcirculation are caused by the deposition of fibrin strands resulting from intravascular activation of the coagulation system (see the discussion of disseminated intravascular coagulation [DIC] in Chapter 28).

In MAHA, the mechanical process leading to fragmentation of the red cells occurs as the blood flow forces the cells to negotiate a blood vessel whose lumen is restricted by microthrombi.⁷⁰ The red cells are physically torn as they are forced along the narrow confines of the blood vessel. Schistocytes and other poikilocytes are seen on the peripheral blood smear, as well as decreased platelets in some cases. The degree of hemolysis correlates with the amount of thrombosis present.⁷¹ In addition to the intravascular destruction of the red cells, the fragments produced lack deformability, leading to an increase in extravascular hemolysis.

In addition to DIC, MAHA may be associated with invasive carcinoma, malignant hypertension, cavernous hemangiomas, complications of pregnancy, kidney or liver transplantation, and bone marrow transplantation.⁷² Manifestations of MAHA are also prominent in two related clinical entities: hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (see Chapter 26).

MAHA can arise in patients experiencing hemolytic uremic syndrome (HUS) and pregnancy complications such as severe preeclampsia and HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets; see the discussion of HUS and HELLP syndrome in Chapter 26).

Chemical and Physical Agents

Exposure to certain chemical and physical agents can affect red blood cells negatively by causing hemolysis. Individuals exposed to the agents covered in this section will have a resulting anemia. A variety of chemical and physical agents are described in this chapter along with their associated mechanisms.

Oxidative Hemolysis

Oxidative stress on the red cell resulting from either drugs or chemicals may affect either the globin chains or the heme group of the hemoglobin molecule. Most oxidizing agents affect the hemoglobin molecule by denaturing the globin chains, producing Heinz bodies, or by oxidizing the heme group, producing methemoglobin (see Chapter 2). Both of these processes will eventually lead to membrane damage and decreased red cell deformability, with eventual extravascular hemolysis in the spleen.⁷³ Glucose-6-phosphate dehydrogenase (G6PD) deficiency increases the susceptibility of

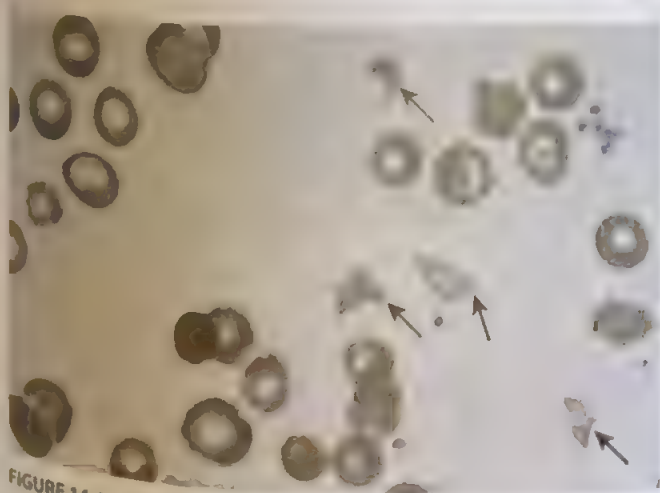


FIGURE 14-14 RBC fragmentation in microangiopathic hemolysis from a patient with a prosthetic cardiac valve (mechanical hemolysis), note the presence of schistocytes (arrows).

red cells to oxidant stress (see Chapter 10). However, strong oxidants can induce hemolysis even in normal individuals. Examples include naphthalene (mothballs), phenol, cresol (Lysol or penetrating oil), and aniline.⁷³

Nonoxidative Hemolysis

Arsenic Industrial processes involving the action of acids and metals may give rise to the production of arsenic gas. Continued exposure to the gas gives rise to intravascular hemolysis with anemia and hemoglobinuria.⁷⁴ Marked methemalbumin formation causes the serum of affected patients to turn a characteristic brown and often masks the presence of any hemoglobinemia. Current Occupational Safety and Health Administration (OSHA) requirements have minimized this hazard in the workplace.

Lead Anemia is associated with either acute or chronic lead poisoning, which includes some component of hemolysis.⁷⁵ The red cells of patients exposed to lead have a shortened survival.⁷⁵ Lead poisoning is usually a problem of young children living in deteriorated housing where they eat chips of lead-based paint. Children affected by lead poisoning may show a normocytic to microcytic, hypochromic blood picture, with classic punctate basophilic stippling (Fig. 14-15).

Adults are more likely to acquire lead poisoning in an occupational setting. These patients commonly present with neurological or renal disease with variable anemia.

Copper Very high levels of copper ions have been associated with intravascular hemolysis. These levels may occur as a result of suicide attempts in which copper sulfate solution is ingested or in Wilson's disease.⁷⁶ The cause of hemolysis is unknown, although it has been shown that high levels of copper ions can affect a number of intracellular enzymes (e.g., pyruvate kinase and hexokinase).⁷⁷ The anemia may be associated with the presence of spherocytes.

Venoms Hemolysis may follow spider bites, bee and wasp stings, and some venomous snake bites. In the United States, bites of the brown recluse spider are known to cause DIC with hemolysis after 24 to 48 hours.⁷⁸ The venomous bites

of pit vipers (rattlesnakes, cottonmouths, water moccasins, and copperheads) also cause DIC with hemolysis.⁷⁹ The phospholipase-A2 in the venom of certain snakes causes hemolysis directly through the action on red cell membranes.⁸⁰

Osmotic Effects

The red blood cell membrane effectively maintains the appropriate osmotic pressure between intracellular and extracellular fluids. Certain situations may disrupt this equilibrium, and red blood cells are at risk of lysis. Situations that can have osmotic effects on red blood cells are described in this section.

Burns Patients who have suffered severe burns to more than 15% of their body may show evidence of intravascular hemolysis. The hemolytic process is thought to result from the direct effect of the heat on the red cells in the affected area. Red cells heated to temperatures in excess of 47°C undergo changes, including fragmentation, budding, and microspherocyte formation; blood collected within 24 hours of such heating shows evidence of these changes (Fig. 14-16). Because the cells are osmotically and mechanically fragile, they are rapidly removed from the circulation, and blood collected after that time is often normal in appearance.

Drowning and Other Water-Related Osmotic Injuries Solute free or hypotonic water can cause osmotic hemolysis. This occurs most commonly in cases of near-drowning in fresh water when sufficient water is inhaled and absorbed through the lungs to cause a decrease in plasma osmolality with some degree of hemolysis.⁸¹ This can also occur when hypotonic solution or distilled water is accidentally used in hemodialysis or as an intravenous fluid. Similarly, distilled water used as an irrigant during urological procedures can enter the circulation through the raw tissue bed in amounts sufficient to cause hemolysis.

Acquired Membrane Disorders

A number of mechanisms can be implicated in producing changes to red cell membranes, which can result in the shortened life span of the cell. Any change that

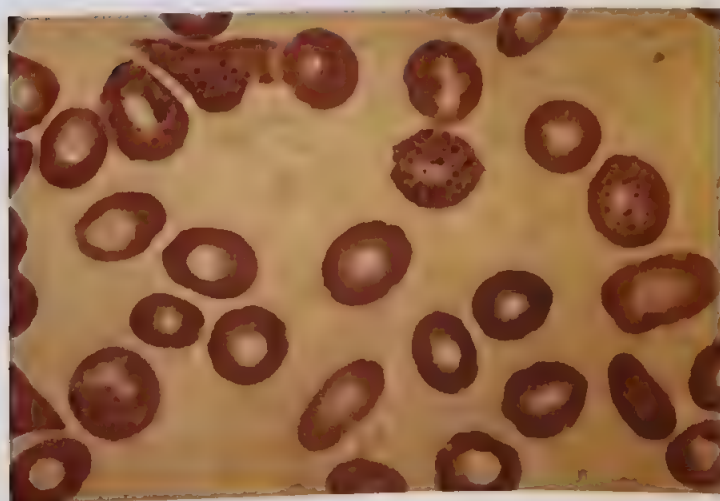


FIGURE 14-15 Peripheral blood from a patient with lead poisoning. Note the normocytic, hypochromic red cells, with the classic punctate basophilic stippling.

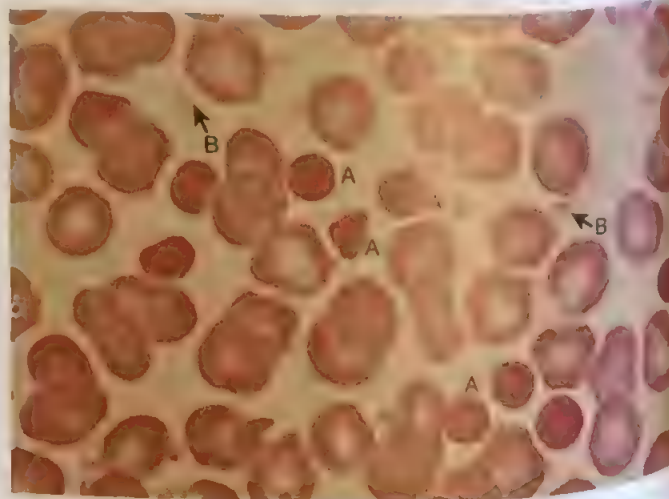


FIGURE 14-16 Peripheral blood from a patient with extensive burns. Note the typical (A) microspherocytes and (B) membranous fragments. (From Bell A. Hematology. In Listen, Look and Learn. Bethesda, MD: Health and Education Resources, Inc., with permission.)

compromises the red cell's deformability or its resistance to oxidative stress can potentially contribute to a hemolytic process. For example, spur-cell anemia, observed primarily in patients with alcoholic cirrhosis, is a condition in which the red cells assume a characteristic spherical shape with a number of fine, fingerlike spike projections (acanthocytes) (Fig. 14-17). Lipid disorders can also result in a loss of red cell deformability. In abetalipoproteinemia, the cells also assume the classic shape of acanthocytes (Fig. 14-18). In end-stage renal disease, many of the cells take on the appearance of burr cells or echinocytes (Fig. 14-19), which have numerous small spines over their entire surface. Other conditions in which echinocytes are seen include pyruvate kinase deficiency and bleeding associated with peptic ulcer disease.

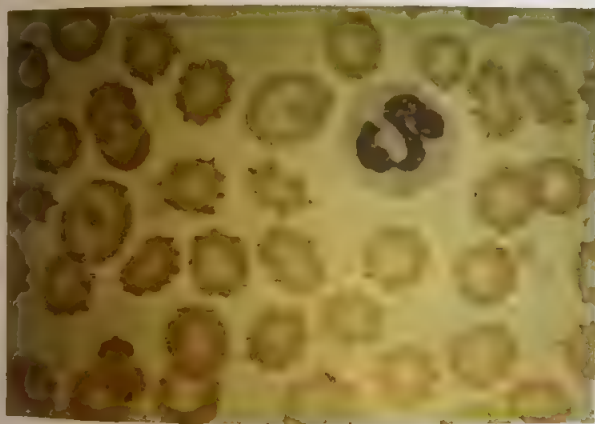


FIGURE 14-17 Spur-cell anemia (acanthocytosis) associated with severe liver disease.

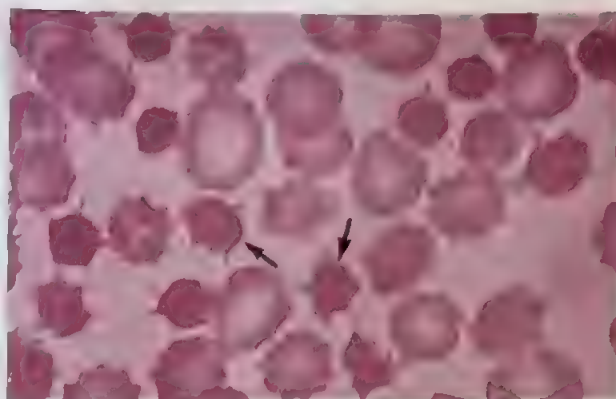


FIGURE 14-18 Acanthocytosis from a patient with abetalipoproteinemia

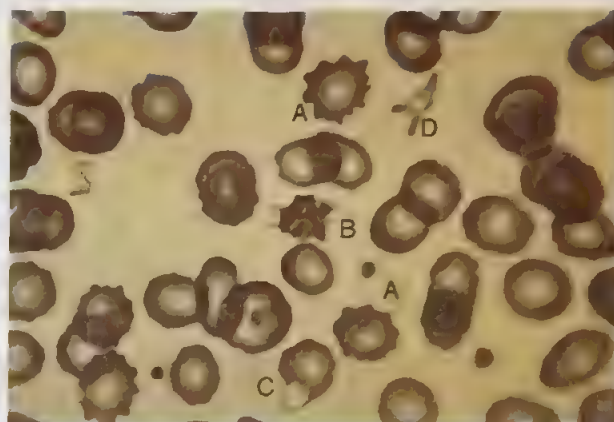


FIGURE 14-19 Renal disease (peripheral blood). Note the presence of (A) burr cells, (B) thorn cell, (C) blister cell, and (D) schistocyte. (From Bell A. Hematology. In Listen, Look and Learn. Bethesda, MD: Health and Education Resources, Inc., with permission.)

SUMMARY CHART

- Complement is a group of serum proteins that interact with each other to bring about complement-dependent cell-mediated lysis.
- Intravascular hemolysis occurs when antibodies bind to antigenic determinants on red cells and activate the classical complement pathway.
- Antibody-dependent cellular cytotoxicity (ADCC) is a form of direct (intravascular) lysis of immunoglobulin-coated cells; effector cells contain receptors for IgG1 and IgG3, and complement proteins C3b and iC3b, which facilitate cell lysis.
- Hemoglobinemia, hemoglobinuria, and decreased haptoglobin levels are common findings in intravascular hemolysis.
- Extravascular hemolysis is the phagocytosis of red cells by fixed phagocytes within the mononuclear phagocyte system (MPS); the two major organs of the MPS are the spleen and liver.
- Common laboratory findings in extravascular hemolysis may include the presence of spherocytes, increased serum indirect bilirubin, and urine urobilinogen.
- In alloimmune hemolytic anemia, patients produce alloantibodies to foreign red cell antigens introduced through transfusions, pregnancy, or organ transplantation.
- In autoimmune hemolytic anemia (AIHA), patients develop antibodies to their own red cell antigens.
- In drug-induced hemolytic anemia, patients produce antibodies directed at a particular drug, its metabolites, or red cells coated with the drug; the four major drug-induced mechanisms include immune complex, drug adsorption, membrane modification, and methylidopa-induced mechanism.
- Acute hemolytic transfusion reactions are characterized by acute intravascular hemolysis and associated with the ABO blood group antibodies.

SUMMARY CHART—cont'd

- A delayed hemolytic transfusion reaction is characterized by exposure to red cell antigens other than the ABO blood group; the reaction may occur from 2 to 10 days after transfusion and is generally the result of an anamnestic response to transfused red cells.
- Hemolytic disease of the fetus and newborn (HDFN) is an immune hemolytic disorder caused by maternal-fetal blood group incompatibility; maternal IgG antibodies with ABO, Rh, or other blood group specificity cross the placenta and destroy antigen-containing fetal red cells.
- Warm autoimmune hemolytic anemia (WAIHA) accounts for 70% of autoimmune hemolytic anemias, and the DAT is positive for both IgG and C3d in 67% of cases, in 20% with IgG alone, and in 13% with C3d alone.
- The specificity of normal cold autoantibodies includes anti-I, anti-H, and anti-IH, which react at temperatures from 4°C to 22°C.
- Cold agglutinin disease (CAD) represents 16% of autoimmune hemolytic anemias; it occurs at a wide thermal range (4°C to more than 30°C); antibody specificity is an IgM toward anti-I, anti-i, and anti-Pr.
- Secondary CAS, caused by infections to *Mycoplasma pneumoniae* and infectious mononucleosis, has antibody specificity toward anti-I and anti-i, respectively.
- The four species of malaria that can infect humans through the mosquito vector are *Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*; *P. falciparum* causes severe disease with an often fatal course.
- Bartonellosis is caused by the organism *Bartonella bacilliformis* and is transmitted by the sand fly; the disease is characterized by a hemolytic phase and a tumor phase.
- *Microangiopathic hemolytic anemia* refers to a group of disorders characterized by fragmentation of the red cells as they pass through abnormal arterioles, resulting in intravascular hemolysis.
- A variety of chemical and environmental agents can cause hemolysis of red cells. These include oxidating agents, arsenic, lead, copper, and insect or snake venoms.

CASE STUDY 14-1

A 26-year-old Caucasian woman diagnosed with rheumatoid arthritis came to the emergency department with complaints of weakness and shortness of breath. Laboratory findings revealed a severe normocytic anemia. The patient had received many red cell transfusions over the past 8 years, but she had not received any transfusions in over a year. The peripheral blood smear demonstrated mild anisocytosis, spherocytosis, and moderate polychromasia. The crossmatch and antibody screen tests were weakly positive at the antiglobulin phase. On further testing, the patient's direct antiglobulin test (DAT) was also weakly positive in polyspecific and IgG phases. The effort to obtain compatible blood for transfusion was complicated because the patient's serum reacted weakly with all cells tested.

QUESTIONS

1. Would you classify this patient's anemia as immune or nonimmune?
2. Do you think the patient's red blood cells are hemolyzing intravascularly or extravascularly? Why?
3. What hemolytic condition is most likely?
4. What may the laboratory findings in this hemolytic condition include?

ANSWERS

1. Immune because the DAT indicates RBCs are coated with IgG and complement.
2. Extravascular hemolysis, because of the presence of spherocytes and the patient does not have hemoglobinuria.
3. Warm autoimmune hemolytic anemia, secondary to rheumatoid arthritis.
4. Laboratory findings in patients with warm autoimmune hemolytic anemia may include increased reticulocyte count, decreased hemoglobin, increased serum lactate dehydrogenase, increased indirect serum bilirubin (product of red cell catabolism), and decreased serum haptoglobin level.

CASE STUDY 14-2

A 38-year-old man was diagnosed with pneumonia 3 weeks previously. He returned to the hospital because he was experiencing severe weakness and shortness of breath on slight exertion. The patient was pale and jaundiced. Laboratory findings revealed severe normocytic anemia. The peripheral blood smear demonstrated marked agglutination of erythrocytes. The crossmatch and antibody screen tests were both strongly positive at the room temperature phase (20°C). On further testing, the patient's DAT was also strongly positive, with complement proteins.

QUESTIONS

1. A patient's red cells agglutinating at room temperature could be indicative of which hemolytic condition?
2. What other laboratory test values may be affected by the agglutination of RBCs? What is the remedy?
3. What laboratory test is the gold standard for diagnosing this condition?
4. What is the treatment for this patient?

ANSWERS

1. Cold agglutinin syndrome. This could be secondary to the patient's pneumonia (probably *Mycoplasma*).
2. Certain complete blood count (CBC) values from an automated instrument will be erroneous because of the agglutination of RBCs at room temperature. The blood should be warmed to 37°C and rerun through the instrument.
3. The Donath–Landsteiner test.
4. This cold agglutinin syndrome would be expected to be self-limited; meanwhile the patient should avoid the cold.

CASE STUDY 14-3

A 50-year-old African American man had recently returned from a trip to Africa to visit relatives. He complained of headaches, fatigue, and general malaise over the past 2 weeks. The patient then developed a high fever followed by severe chills, which persisted for approximately a day. The fever and chills subsided, and the patient thought he was getting better until last night, when he experienced another bout of fever and chills. Laboratory findings revealed the patient was anemic and slightly leukopenic. Odd, ring-like inclusions were seen in approximately 10% of his red cells, along with rare blue, crescent-shaped forms that appeared to be extracellular. On further testing, the DAT was negative. The crossmatch and antibody screening tests were also negative.

QUESTIONS

1. What is the most likely diagnosis?
2. What species might be the causative agent?
3. What other laboratory tests should be requested to aid in the diagnosis?
4. What is the vector for this disease?
5. What type of hemolysis occurs as a result of the infection?

ANSWERS

1. Malaria.
2. Most likely *Plasmodium falciparum*.
3. Thick smear preparation, stained with Giemsa or Wright's stain; it is best to use fingerstick blood rather than anticoagulated blood.
4. The vector is the *Anopheles* mosquito.
5. Hemolysis in malaria occurs intravascularly as a result of direct red cell destruction by the parasite.

REVIEW QUESTIONS

- Which of the following is a mechanism of immune hemolysis?
 - IgG or IgM antibodies that activate the classical complement pathway
 - Antibody-dependent cellular cytotoxicity (ADCC) mediated by B cells, monocytes/macrophages, and granulocytes
 - Complete or partial phagocytosis of erythrocytes by fixed macrophages
 - Deficient erythrocytic enzymes fail to protect the cell
- Which would best distinguish hemolytic anemia caused by immune mechanisms from other hemolytic anemias?
 - Presence of spherocytes on peripheral blood film
 - Increased reticulocyte count
 - Enlarged spleen
 - Positive DAT
- Which is true concerning autoimmune hemolytic anemia?
 - Majority of cases are of the "cold" type
 - Seen in transfusion reactions
 - Is demonstrated in hemolytic disease of the fetus and newborn
 - Antibodies are produced against one's own erythrocyte antigens
- What is the process in which the immune system produces antibodies to foreign red cell antigens introduced into their circulation through transfusion, pregnancy, or organ transplantation?
 - Alloimmune hemolytic anemia
 - Autoimmune hemolytic anemia
 - Drug-induced immune hemolytic anemia
 - None of the above
- What causes hemolytic disease of the fetus and newborn (HDFN)?
 - Maternal IgG antibodies, formed as a result of a previous blood exposure or pregnancy, cross the placenta and attach to fetal cells.
 - Fetal IgG antibodies cross the placenta and attach to maternal red cells.
 - Maternal IgM antibodies, formed as a result of a previous blood exposure or pregnancy, cross the placenta and attach to fetal cells.
 - Fetal IgM antibodies attach to fetal red cells and cross the placenta to enter the mother's circulation.
- Which is *not* a characteristic of warm autoimmune hemolytic anemia?
 - Variable anemia
 - Reticulocytosis and spherocytosis
 - Positive result for Donath-Landsteiner test
 - DAT result usually positive for both IgG and C3d
- What is a feature of cold agglutinin syndrome?
 - Usually an IgG antibody
 - Reticulocytosis and positive DAT
 - Predominantly in younger patients
 - Reacts only on temperatures below 22° C
- Which of the following describes a characteristic of acute hemolytic transfusion reactions?
 - The causative antibody is typically IgG.
 - Patients may not present with symptoms for days to weeks.
 - Causative antibodies are most often those associated with ABO blood groups.
 - Red blood cell lysis is exclusively extravascular in nature.
- Which of the following is not implicated with nonimmune hemolytic anemia?
 - Leukemia
 - Mechanical, chemical, and physical agents
 - Acquired membrane disorders
 - Infections
- Which of the following is an acquired membrane disorder causing a nonimmune hemolytic anemia?
 - Mycoplasma pneumoniae*
 - Clostridium perfringens*
 - Liver Disease
 - Staphylococcus aureus*
- Which of the following, as measured via an automated hematology instrument, would most likely be affected by a cold agglutinin?
 - Hemoglobin
 - Hematocrit
 - Platelet count
 - Leukocyte count
- Which of the following drugs causes a hemolytic anemia resulting from production of "true autoantibodies" rather than antibodies to the drug or to the drug-erythrocyte complex?
 - Penicillin
 - Cephalosporin
 - Aldomet
 - Quinidine
- Which cause for hemolytic anemia is typically seen in children living in old, deteriorating homes?
 - Copper
 - Lead
 - Mercury
 - Arsenic

REVIEW QUESTIONS—cont'd

14. Microangiopathic hemolytic anemia is a hemolytic anemia caused by which of the following?
 - a. Autoimmune processes
 - b. Extracellular infection
 - c. Drugs
 - d. Mechanical impacts
15. Which of the following disorders may be associated with warm autoimmune hemolytic anemia?
 - a. Infectious mononucleosis
 - b. Systemic lupus erythematosus
 - c. Myelogenous leukemia
 - d. Mycoplasma pneumonia
16. In paroxysmal cold hemoglobinuria (PCH)
 - a. IgM autoantibodies are involved
 - b. Complement is not activated
 - c. Hemolysis is extravascular
 - d. IgG autoantibodies are involved

See answers at the back of this book.

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Anemia Associated With Systemic Diseases

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CHAPTER OUTLINE

Introduction

Anemia of Chronic Kidney Disease

Etiology and Pathophysiology
Clinical Findings
Laboratory Evaluation
Treatment

Anemia of Liver Disease

Etiology and Pathophysiology
Clinical Findings
Laboratory Evaluation
Treatment

Anemia of Endocrine Disease/Disorders

Diabetes
Adrenal Insufficiency

Thyroid Disease

Hyperparathyroidism
Hypogonadism
Pituitary Dysfunction

Myelophthitic Anemia

Etiology and Pathophysiology
Clinical Findings
Laboratory Evaluation
Treatment

Anemia Associated With Viral Infections

SARS-CoV-2 and COVID-19
Etiology and Pathophysiology
Clinical Findings
Laboratory Evaluation
HIV and AIDS

Anemia of Prematurity

Etiology and Pathophysiology
Clinical Findings
Laboratory Evaluation
Treatment

Summary Chart

Case Study 15-1

Case Study 15-2

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 15-1** Analyze the pathophysiology of anemia of chronic kidney disease.
- 15-2** Identify the treatment for anemia of chronic kidney disease.
- 15-3** Evaluate the characteristics of the red blood cells in anemia of liver disease.
- 15-4** Differentiate the etiology of anemia of kidney disease from anemia of liver disease.
- 15-5** Compare the mechanisms involved in myelophthitic anemia to other hypoproliferative anemias.
- 15-6** Assess the laboratory findings associated with anemia of endocrine dysfunction.
- 15-7** Summarize the proposed pathophysiology of anemia caused by viral infections.
- 15-8** Assess the pathophysiology of anemia of prematurity.

Anemias associated with inflammation or chronic systemic diseases are commonly hypoproliferative anemias, because they are not accompanied by an appropriate proliferative response by the bone marrow. This lack of bone marrow response is evident by analysis of the patient's absolute reticulocyte count having a low or normal value. It is important for clinicians and laboratory professionals to recognize the characteristics of these anemias in patients with systemic diseases and to understand the laboratory studies required to diagnose them. Anemia in a patient without other clinical symptoms at presentation could be the first indication of a systemic disease, such as viral infection or malignancy. Likewise, a sudden change in the complete blood count (CBC) of a patient who has been diagnosed with an inflammatory or systemic disease might indicate a new complication. For this reason, a CBC should be part of the initial laboratory evaluation.

The common theme of the anemias described in this chapter is the role of systemic disorders (i.e., nonhematologic disorders) by suppressing red blood cell production in the bone marrow, resulting in a hypoproliferative anemia. Anemia of Chronic Inflammation is discussed in Chapter 7.

Anemia of Chronic Kidney Disease

Renal disease is associated with a wide variety of hematologic abnormalities. These include anemia, abnormal platelet function, abnormal white blood cell function, and coagulopathy. The latter usually results from the direct effect of uremia on platelet and coagulation factor function. Anemia almost invariably accompanies significant chronic renal insufficiency or renal failure.

Etiology and Pathophysiology

The anemia of chronic kidney disease is caused by:

- Inadequate EPO production by damaged kidneys
- A slightly to moderately reduced RBC life span (eryptosis- programmed cell death of RBCs) induced by uremic toxins^{1,2}
- Suppression of erythropoiesis by inflammation and uremic toxins that accumulate in renal failure and chronic kidney disease

Other contributing factors may include nutritional disorders (B₁₂, folate deficiency, iron deficiency), hormonal disorders, hemolysis, inflammation, and other underlying disorders.³ The mechanisms involved in anemia of chronic kidney disease are outlined in Box 15-1.

The primary cause of anemia of chronic kidney disease is decreased production of EPO by failing kidneys. Despite this, it is not recommended to routinely evaluate EPO levels to diagnose or monitor patients with chronic renal disease.⁴ Measured EPO levels may be decreased, normal, or increased. Normal or increased levels would still be considered "abnormal" for the degree of anemia present. Thus, the EPO production by the diseased kidneys is insufficient for the severity of the anemia present, and administration of EPO-stimulating agents becomes a primary treatment for anemia of chronic kidney disease in patients with adequate iron status or concurrent iron supplementation.^{2,4,5}

ADVANCED CONTENT

There is also evidence that points to additional mechanisms in the anemia of renal disease beyond a decreased production of EPO. Some patients continue to have anemia despite the use of EPO-stimulating agents and iron

BOX 15-1 Mechanisms Involved in Anemia of Chronic Kidney Disease

Inadequate EPO Produced by Damaged Kidneys

- EPO is inadequate for the degree of anemia
- Decrease in erythroid precursors in the bone marrow

Suppression of erythropoiesis by uremic toxins

- Dialyzable toxins such as polyamines
- "Middle molecules" of inflammation

Reduced RBC Life Span

- Extracorporeal defect related to uremic toxins

Other Complicating Causes of Anemia

- Inflammation (ACI)
- Hyperparathyroidism
- Blood loss (related to dialysis or GI hemorrhage)
- Iron deficiency
- Folate deficiency
- Dilutional anemia related to fluid retention

supplementation. Uremic toxins in the plasma of patients with end stage renal disease (ESRD) and chronic kidney disease (CKD) have been shown to have increased expression of phosphatidylserine on the outside membrane of the RBCs. This phospholipid flops from the inner membrane to the outer membrane of aging RBCs as a marker for cell death and can also serve to indirectly activate platelets as microparticles release from the RBCs.² Serum taken from patients before dialysis has been used to demonstrate the increase in eryptosis, whereas serum taken postdialysis has not been shown to increase eryptosis.¹ The increase of eryptosis in renal patients may also contribute to additional complications including increased cardiac risk owing to the adhesion of the eryptotic cells to the endothelium and exposure of the phosphatidylserine that can activate platelets, which could be compounded by the use of EPO to produce more RBCs that would be prone to eryptosis, thus adding to the downstream effects.^{1,2} Chronic inflammation has been associated with a decline in renal function. The pathophysiology of this chronic inflammatory state has yet to be elucidated, though it is thought to have many factors including immune dysfunction, alteration of the gut microbiome, retention of uremic toxins, and oxidative stress factors.³ Elevations in IL6 have been associated with worse outcomes. Uremic retention solutes have been shown to include "middle molecules" including cytokines and pro-inflammatory mediators (e.g., IL6, IL-1 β , TNF, β 2-microglobulin, parathyroid hormone, ghrelin [a hunger hormone released by the stomach], etc.), which make up 23% of the uremic toxins present and have been shown to increase in proportion with the severity of renal dysfunction.³ This inflammatory state may lead to the increased levels of hepcidin seen in patients with CKD. The increased hepcidin levels may be the cause of impaired erythropoiesis in the presence of adequate EPO. This iron-restricted anemia presentation may further complicate differential diagnosis of ACI or anemia of CKD. A large portion of these "middle molecules" are removed through dialysis, whereas larger protein-bound molecules such as indoxyl sulfate are not removed sufficiently.^{2,3} The indoxyl sulfate has many toxic effects including vascular damage but may also have a direct inhibitory effect on the expression of the EPO receptors in erythroid precursors, which impair the EPO synthesis in the kidney by preventing the activation of hypoxia inducible factors.² Additionally, other protein-bound uremic toxins have been shown to directly inhibit erythropoiesis by decreasing proliferation and maturation of erythroid precursors.²

Ultimately, the uremic toxins seen in patients with CKD modulate anemia by decreasing the production of EPO, increasing eryptosis, and by inhibiting erythropoiesis in the bone marrow through the reduction of EPO receptors. In addition, these uremic toxins decrease proliferation and maturation of erythroid precursors, and modulate hepcidin regulatory pathways with iron restriction and persistent inflammation mediators.²

Clinical Findings

Patients with chronic kidney disease should be screened for anemia at least annually as anemia develops in virtually all patients with CKD.⁴ CKD can be a complication of diabetes, inflammation, hypertension, renal fibrosis, and other chronic diseases. It can remain undetected as patients are often asymptomatic until the dysfunction progresses.^{5,6}

Laboratory Evaluation

The anemia of chronic kidney disease may be more severe depending on the severity and duration of the renal dysfunction and is usually normocytic with a decreased or (inappropriately) normal reticulocyte count classifying it as a hypoproliferative anemia. On peripheral blood films, erythrocytes are normocytic and normochromic; burr cells, sometimes seen in uremia, may also be present (Fig. 15-1).

CRITICAL THINKING QUESTION

15-1 Would you expect the erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) to be normal, increased, or decreased in anemia of chronic kidney disease?

See answers to all Critical Thinking Questions at the back of this book.

Treatment

The current guidelines for treatment of anemia in chronic kidney diseases begins with the evaluation of iron stores. If the patient is iron deficient, oral or IV iron supplementation is recommended along with the EPO-stimulating agents. Dialysis will help decrease the “middle molecules” but not the protein-bound molecules. The use of IV iron should be used with caution, particularly in the context of patient needing transfusions to avoid the potential for iron overload. The serum ferritin and serum transferrin saturation should be monitored every 1 to 3 months for elevations that would be indicative of iron overload.^{4,7}

Anemia of Liver Disease

A wide array of hematologic disorders is encountered in patients with liver disease. The morphology and degree of

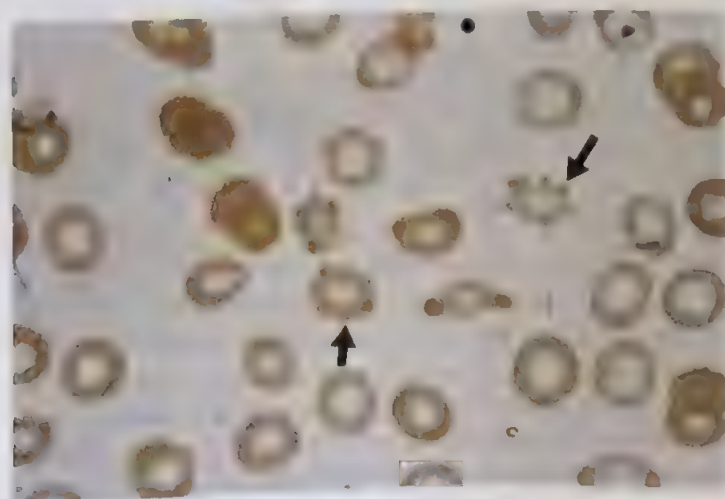


FIGURE 15-1 Peripheral blood from a patient with renal disease. Note the burr cells (arrows). (From Bell A. Hematology. In Listen, Look and Learn. Bethesda, MD: Health and Education Resources, Inc., with permission.)

anemia differ somewhat depending on the cause and duration of the liver disease and on the presence or absence of other associated effects (Box 15-2). Most cases of anemia associated with liver disease are seen in patients with chronic liver disease. One of the most common causes of liver disease and liver damage is excessive alcohol ingestion.

Etiology and Pathophysiology

Chronic liver disease brings to mind more problems with hemostasis than with complete blood cell counts (CBC); however, anemia is often present (in about 75% of patients) through a variety of mechanisms.⁸ Depending on the underlying cause of anemia, it may present as macrocytic, normocytic, or microcytic. In chronic liver disease, the anemia may be severe but overall does not directly correlate with the severity of liver disease. It can be caused by complications related to liver damage such as bleeding, hemolysis, acquired hemolytic anemias (spur cell), viral hepatitis complications, bone marrow toxicity, and malnutrition.^{9,10}

Clinical Findings

Patients with chronic liver disease, liver failure, and end-stage cirrhosis have decreased capacity for the production of coagulation factors by the liver, especially the vitamin K-dependent factors (see Chapter 25, Hemostasis, and Chapter 27, Disorders of Secondary Hemostasis). This may be a cause of chronic blood loss or severe bleeding associated with hemostatic challenges such as surgery. The anemia can be normocytic and normochromic. However, increasing macrocytes often cause the MCV to be modestly elevated. Patients with chronic liver disease may also have hypersplenism caused by portal hypertension and, in some cases, thrombosis of the splenic vein.¹ Hypersplenism is a cause of platelet sequestration leading to thrombocytopenia and associated chronic bleeding tendencies, especially gastrointestinal and mucosal surface bleeding. Hypersplenism can also lead to decreased red blood cell survival as RBCs are trapped in the reticuloendothelial system.

BOX 15-2 Mechanisms of Anemia in Liver Disease

Direct Effects

- Toxic effects of ethanol (dyserythropoiesis, folate deficiency)
- Vacuolization of marrow hematopoietic precursor cells
- Decreased marrow cellularity
- Macronormoblastic changes NOT associated with folate deficiency or vitamin B₁₂ deficiency
- Acute and chronic blood loss
- Gastrointestinal bleeding (Alcoholism)
- Liver disease-associated coagulopathic states
- Viral suppression of erythropoiesis (Hepatitis)

Indirect Effects

- Dilutional anemia
- Hypersplenism, erythrocyte sequestration
- Hemolytic anemia
- Spur-cell anemia (acanthocytosis)
- RE macrophage activity
- Malnutrition
- Protein, folate, iron, and vitamin deficiencies
- Anemia of chronic inflammation

Liver disease caused by hepatitis can also cause aplastic anemia with viral infection of the bone marrow and erythrocyte precursors. This infectious process can directly affect the cells and also initiate the immune response, which inhibits erythropoiesis through INF- γ as described in ACI in Chapter 7.⁸

Excessive alcohol consumption can contribute to a hypoproliferative anemia in several ways. Alcohol has direct toxic effects on red blood cell precursors and bone marrow cellularity. There is a direct inhibition of hematopoietic precursors that can result in a variety of cytopenias, including leukocytopenia, thrombocytopenia, and anemia.^{11,12} The RBC lineage seems to be the most susceptible to this direct inhibition through both alcohol and acetaldehyde (a by-product of alcohol metabolism) and potentially explains the frequency of anemia rather than leukocytopenia in heavy alcohol users.¹² Alcohol consumption is also associated with folate deficiency (even in patients with proper nutrition).^{8,11} Folate deficiency may be associated with direct effects of alcohol on folate metabolism, including increased urinary folate excretion, decreased liver storage, and impaired intestinal absorption.¹³ Additionally, chronic alcohol consumption has been associated with changes in iron metabolism leading to increased iron storage and sideroblastic anemia as a consequence of the folate deficiency, iron consumption in alcoholic beverages (e.g., wine), and/or therapies administered.¹³

Laboratory Evaluation

Overall, anemia associated with chronic liver disease alone (without complications) is mild to moderate. It is hypoproliferative, as demonstrated by absent reticulocytosis (decreased number of reticulocytes for the degree of anemia) and may show variable RBC morphology, including moderate numbers of round macrocytes and acanthocytes.

Target cells and acanthocytes are seen on the peripheral blood smear in cases of chronic liver disease and are a consequence of the altered lipid production¹⁴ seen with liver dysfunction (Fig. 15-2 and Chapter 2, The Red Blood Cell). Macrocytosis with the formation of target cells is seen on the

peripheral blood smear in cases of chronic liver disease with a level of obstructive jaundice or hepatocellular cholestasis affecting lipid metabolism because of portal hypertension.⁹

Morphological changes such as vacuolization of erythroblasts and red blood cell precursors in the bone marrow are observed in alcoholic patients.^{13,15} Bone marrow biopsies from alcoholic patients present with megaloblastic changes in the erythrocyte lineage only, ringed sideroblasts, and vacuoles in the erythropoietic precursors and may resemble those from patients with myelodysplastic conditions.¹⁵

Lipid metabolism is negatively affected by liver disease. Decreased lecithin-cholesterol acyltransferase enzyme (LCAT) in severe liver disease is also thought to play a role in the deposition of cholesterol in the RBC membrane due to increased free cholesterol in the plasma.^{13,16,17} Target cells are formed due to increased membrane lipid (cholesterol and phospholipids) content, whereas acanthocytes are due to excess membrane cholesterol (Table 15-1). A rare complication of severe liver disease is a hemolytic anemia caused by rigid membranes of the acanthocytes (spur cell anemia) decreasing the deformability of the cell as it passes through the spleen.^{9,10,17}

CRITICAL THINKING QUESTION

15-2 In consideration of a hypoproliferative anemia, would you expect to see polychromasia on the peripheral blood smear?

ADVANCED CONTENT

Macrocytes in liver disease have different morphological features depending on their cause. Macrocytosis is often seen in patients with cirrhosis, obstructive jaundice, and other types of liver disease without the presence of anemia.¹⁸ These macrocytes are due to abnormal membrane cholesterol and phospholipid content and reflect the lipid metabolism dysfunction in liver disease. The survival of these macrocytic red cells is not typically decreased. These macrocytes are not, however, the macroovalocytes seen in megaloblastic anemia, and these patients do not have other markers of megaloblastic changes (refer to Chapter 8).⁸ This type of macrocytosis does not respond to vitamin B₁₂.

TABLE 15-1 RBC Morphology in Liver Disease

Morphology	Pathophysiology
Target cells	↑ Membrane lipids
Spur cells	↑ Membrane lipids
Macrocytosis	↓ Folate, ↑ membrane lipids
Reticulocytosis	Anemia
Macronormoblasts*	↑ Membrane lipids

*Bone marrow erythroblasts.

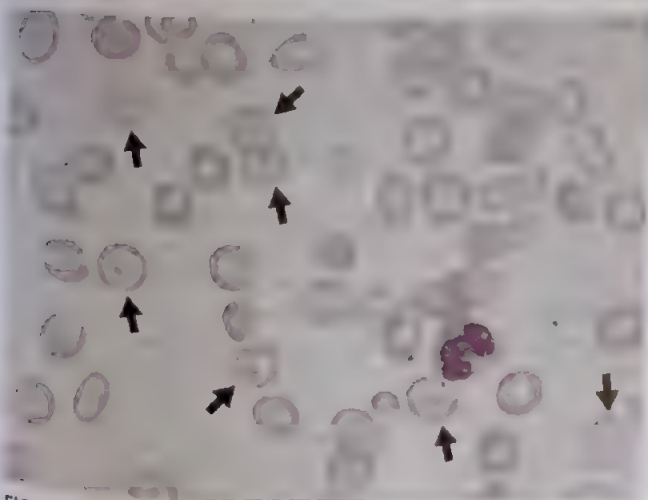


FIGURE 15-2 Peripheral blood smear from an individual with severe liver disease. Target cells (arrows) are caused by altered lipid metabolism with subsequent effects on red blood cell membrane.

or folate therapy but usually resolves only with improvement of liver function and decreased severity of disease. However, macroovalocytes can be observed in some end-stage liver disease patients due to the complication of megaloblastic anemia caused by underlying folate deficiency due to poor nutrition, alcohol consumption, or increased RBC need (accelerated maturation).¹⁸

Treatment

Correction of any dietary deficiencies (lack of iron or folate) can help the anemia of chronic liver disease. However, resolution of the liver disease itself corrects the macrocytosis, acanthocytosis, and anemia. In most instances, advanced liver disease and hypersplenism (when present) are irreversible. In these cases, dietary supplementation (iron, vitamin B₁₂, and folate) can be efficacious in preventing any hematologic sequelae; however, bizarre red cell morphology and low-grade anemia may persist due to the liver disease alone.

Anemia of Endocrine Disease/Disorders

The spectrum of endocrine diseases and their effects on metabolism is wide. Patients with endocrine disease can have disturbances in protein synthesis, energy metabolism, growth, and mineral metabolism. Many of the same hormones involved in endocrine disorders are also involved in the regulation of hematopoiesis. Diseases of the adrenal, thyroid gland, parathyroid glands, gonads, and pituitary gland are associated with the presence of anemia. Many of these often present together in a polyendocrinopathy or autoimmune polyglandular syndromes (APS). Those presenting with APS are at a higher risk for B₁₂ deficiency or pernicious anemia due to atrophic or autoimmune gastritis and a decrease in intrinsic factor.^{19,20} While anemia is common in many of these endocrine disorders or combination of disorders, it is not diagnostic and can be complicated by comorbidities (iron depletion, bleeding, nutrition, autoimmune gastritis leading to pernicious anemia, etc.).

Diabetes

Diabetes is considered the most common endocrine disorder in the United States with an estimated prevalence of 13% in U.S. adults; 34.5% of U.S. adults are prediabetic with increased cases every year.²¹ Type 1 diabetes is marked by deficiencies in insulin production, while type 2 diabetes reflects a more insulin-resistant phenotype. Type 2 diabetes is more common in the United States, and risk increases with obesity and age.²¹

The evolution of anemia in diabetes (both type 1 and type 2) has been explored as a possible risk factor for diabetic complications, including nephropathy and cardiovascular disease.^{22,23} A decreased production in EPO or inappropriate response of EPO for compensation of anemia (hypoproliferative response) has been shown to be one of the predominant mechanisms of anemia in diabetics. In type 1 diabetes, anemia is often associated with autoimmune causes leading to decreased absorption (due to gastritis) of iron or B₁₂.^{23,24} Type 2 diabetes has several mechanisms including the inflammatory response, deficiencies

in iron and EPO, decreased life span of RBCs, and even a decreased EPO response due to reduced functional EPO or glycation of the EPO receptor.²³⁻²⁵

The mechanisms of the inflammatory response include an increase in proinflammatory cytokines (e.g., IL1, IL6, TNF α) as seen with ACL. As hyperglycemic states persist, the increased circulating sugar and glycation products impair the function of hypoxia-inducible factor, cause glycation of EPO receptors thereby inhibiting erythropoiesis, and add to the inflammatory response.^{23,24}

Treatments for type 2 diabetes such as metformin, thiazolidinediones, and angiotensin-converting enzyme inhibitors have also been shown to contribute to anemia in diabetics.^{23,26} Treatments for anemia associated with type 2 diabetes are to treat the underlying disease, increase glycemic control, and decrease inflammation.²⁵

Adrenal Insufficiency

Primary chronic adrenocortical insufficiency, or Addison disease, has several causes. The most common is autoimmune adrenalitis.²⁶ It can present alone, but about half of the cases of autoimmune adrenalitis present as part of autoimmune polyglandular syndromes.²⁰ Patients with adrenocortical insufficiency may develop weakness and fatigability because of corticosteroid or steroid hormone insufficiencies. Addison disease occurs in 1 and 20,000 in the United States, and human leukocyte antigen (HLA)-B8, -DR3, and -DR4 are associated with increased risk.²⁶

When autoimmune Addison disease presents with other autoimmune endocrine diseases (including diabetes mellitus, hypoparathyroidism, hypogonadism, and autoimmune thyroid disorders), the autoantibodies cause destruction of the gland or organ resulting in subsequent hypofunction. Patients may develop pernicious anemia through destruction of gastric lining cells in autoimmune gastritis leading to decreased or absent production of intrinsic factor—the binding factor released by stomach-lining cells that binds vitamin B₁₂ from the diet and permits its absorption from the gastrointestinal tract at the level of the ileum (small intestine). The etiology of the anemia in this instance is vitamin B₁₂ deficiency similar to nutrient deficiency megaloblastic anemia (see Chapter 1: Megaloblastic Anemias). Treatment with intramuscular injections of vitamin B₁₂ may be helpful in this case as a means of bypassing the gastrointestinal tract, thereby guaranteeing entry of vitamin B₁₂ into the body by the intramuscular as opposed to the oral route.

Idiopathic adrenalitis may cause isolated adrenocortical insufficiency (glucocorticoid/steroid deficiency) resulting in a mild, normocytic, normochromic hypoproliferative anemia (no reticulocytosis). The anemia may not be evident because of the accompanying decrease in plasma volume, which masks concurrently with the adrenal insufficiency and may mask the mild anemia.

The etiology of the anemia of adrenal insufficiency is unclear but indicates some contribution to normal erythropoietic activity by glucocorticoids as they have been shown to be vital in the self-renewal of burst-forming unit erythroid progenitors (BFU-E).²⁷ Treatment with an exogenous source

corticosteroids (typically oral prednisone or hydrocortisone) will usually correct this mild anemia.²⁶

Thyroid Disease

Thyroid hormones affect the basal metabolic rate changing the rate of oxygen and energy consumption. EPO production by the kidney depends on tissue oxygenation, which is influenced by the metabolic rate and indirectly by thyroid hormones (e.g., tri-iodothyronine [T_3] and tetra-iodothyronine [T_4]). Erythropoiesis will be increased (hyperthyroidism) or decreased (hypothyroidism) based on the demand for oxygen reflecting the increased basal metabolic rate (hyperthyroidism) or decreased metabolic rate (hypothyroidism). Conflicting studies have shown anemia presenting with both hyperthyroidism and hypothyroidism.

Hypothyroidism often presents with a mild normocytic to macrocytic hypoproliferative anemia. The thyroid hormone receptor alpha is expressed by erythroid precursors and has been shown to regulate erythropoiesis. Hypothyroid patients present with decreased erythroid precursors in the bone marrow and decreased serum levels of EPO (most likely due to the decreased metabolic rate and tissue oxygenation).¹⁹

Because of increased erythropoiesis, hyperthyroidism is not as frequently associated with anemia. However, a study evaluating anemia in Graves hyperthyroidism reported a third of the patients presenting with anemia.¹⁹ Mechanisms for anemia in hyperthyroid patients are independent of erythroid hyperplasia of the bone marrow and increased serum EPO levels. Changes in iron metabolism and decreased RBC life span due to increased oxidative stress have been suggested as possible mechanisms.¹⁹

The clinical effect of anemia with hypothyroidism and hyperthyroidism remains under investigation. A recent cohort study showed that subclinical thyroid dysfunction was not a risk factor for developing anemia, as <10% of participants with subclinical thyroid dysfunction developed anemia within 5 years in a follow-up investigation.²⁸ However, other studies have indicated increased reporting of both anemia and hypothyroidism in over half of the participants, with female patients and older patients reporting lower hemoglobin levels.¹⁹ Additional mechanisms for anemia in these patient populations, such as iron deficiency and blood loss, may require more urgent treatment. Iron deficiency anemia (IDA) may mask the presence of hypothyroidism.¹⁹

Hyperparathyroidism

Hypoproliferative anemia occurs in primary hyperparathyroidism in 5% to 30% of patients.^{29,30} The anemia is mild to moderate in degree, normocytic, normochromic, and is hypoproliferative. Anemic patients tend to have more severe hyperparathyroidism with higher levels of parathyroid hormone, serum calcium, and alkaline phosphatase. They are also more likely to have increased severity of bone disease, bone pain and fractures, kidney stones, and bone marrow fibrosis.^{29,30}

The pathogenesis of anemia of hyperparathyroidism is unclear and is most likely multifactorial. Parathyroid hormone (PTH) may decrease proliferation of erythroid precursors in the bone marrow through increased proinflammatory

cytokines (IL6, TNF α), decreased EPO production, and stimulation of fibroblasts through activation of platelet-derived growth factor-alpha (PDGF- α).²⁹⁻³¹ While bone marrow fibrosis is the most likely cause of anemia in these patients, myelophthasic changes (dacryocytes and leukoerythroblastosis) in the bone marrow and peripheral blood are not seen.^{29,30}

The treatment of this anemia involves the treatment of the underlying hyperparathyroidism through parathyroidectomy. Patients undergoing parathyroidectomy generally improve both the anemia and the bone marrow fibrosis.^{29,30}

Patients with renal failure frequently develop secondary hyperparathyroidism. In these patients, it is difficult to sort out the contribution of the anemia of hyperparathyroidism from that of anemia of chronic kidney disease. In addition to the mechanisms of anemia hyperparathyroidism listed previously, PTH is also considered a uremic toxin and can contribute to anemia seen in chronic kidney disease patients. Treatment of underlying secondary hyperparathyroidism in kidney patients with calcimimetics and vitamin D could improve the response to EPO-stimulating agents.³¹

Hypogonadism

Androgens are other hormones that have important roles as stimulants of erythroid activity, both physiologically and therapeutically. For example, the higher normal range for hemoglobin (HGB) concentrations in men compared with women reflects the effect of androgens. Healthy men have HGB levels up to 1.0 g/dL higher than those of healthy women. Men with hypogonadism (as well as boys and elderly men) have hemoglobin levels similar to those seen in adult females. In these instances, serum androgen (i.e., testosterone) levels are decreased while follicle-stimulating hormone and luteinizing hormone levels (i.e., pituitary hormones) are increased in an attempt by the pituitary to stimulate the nonfunctioning gonads. In a mature man with gonadal hypofunction, the decreased androgen levels result in a mild normocytic, normochromic anemia accompanied by a reticulocytopenia that may be reversed with the administration of exogenous androgens.

Androgens seem to promote erythropoiesis in at least two ways: (1) increasing the production of EPO by the kidney and (2) stimulating the marrow in conjunction with EPO. Testosterone has been shown to increase red cell mass directly by suppression of hepcidin, stimulation of the renal production of EPO (unknown mechanism), stimulation of erythropoiesis and EPO sensitivity in erythroid precursors, and conversion of testosterone to estradiol that further regulates hepcidin.³² Androgens also increase 2,3-bisphosphoglycerate (2,3-BPG), increasing oxygen delivery to the tissues and stimulating granulocyte and platelet production (resulting in increased thrombotic risks).³³

Pituitary Dysfunction

In panhypopituitarism, there is a deficiency in five hormones produced by the anterior pituitary: growth hormone, thyroid stimulating hormone (TSH), adrenocorticotrophic hormone (ACTH), prolactin, and gonadotropin.³⁴ These hormones have a direct relationship with erythropoiesis in the bone marrow. As a result, a mild, normocytic, normochromic, hypoproliferative

anemia is seen in patients with hypopituitarism. To correct this anemia, patients usually require the exogenous replacement of thyroid hormone, androgens, and/or estrogens, as well as the administration of corticosteroids.³⁵ Rare cases of pancytopenia have been documented in patients with panhypopituitarism.

A summary of endocrine disorders including the type/morphology of the anemia, pathogenesis, and treatment is outlined in Table 15-2.

Myelophthistic Anemia

Myelophthistic anemia is very common in patients with both hematologic and nonhematologic malignancies (Fig. 15-3). The term “myelophthistic” refers to bone marrow infiltration by malignant cells (including tumors and granulomas) or to marrow replacement by fibrosis (increases in collagen and/or reticulin). The type of abnormality depends on a multitude of factors, including the type of infiltration being fibrous or cellular, the type of cancer, the site or sites in the body involved by the malignancy, the patient’s treatment with chemo- or radiation therapy, and the extent of involvement of the hematopoietic tissues (bone marrow).³⁶ Box 15-3 lists the direct and indirect effects of the mechanisms of myelophthistic anemia.

Etiology and Pathophysiology

When the bone marrow is infiltrated by a malignant tumor, it can lead to anemia known as myelophthistic anemia. Other causes of hypoproliferative anemias, such as CKD and the anemias associated with endocrine disorders, must be ruled out in the differential diagnosis. Additionally, other causes of bone marrow failure such as hematologic malignancies, infection, and drugs should also be considered. In most cases of malignancy involving the bone marrow, some degree of

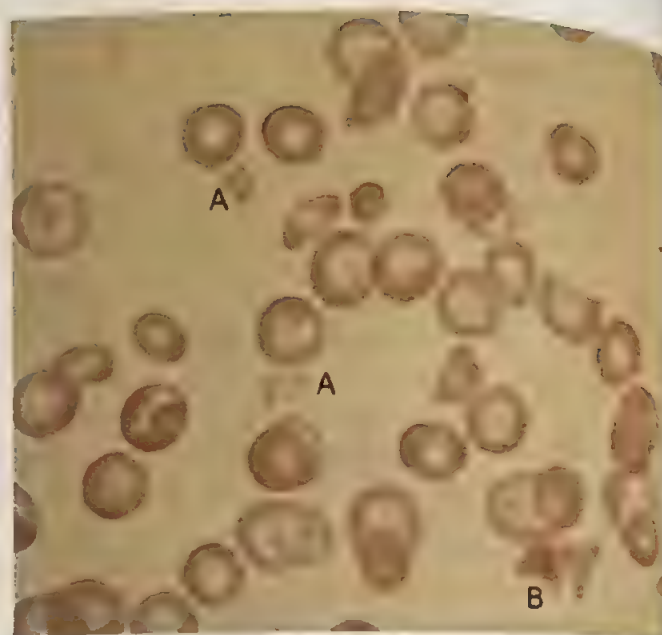


FIGURE 15-3 Peripheral blood from a patient with disseminated carcinoma. Note presence of (A) schistocytes and (B) helmet cells. (From Bell A. Hematology. In Listen, Look and Learn. Bethesda, MD: Health and Education Resources, Inc., with permission.)

reactive, space-occupying fibrosis accompanies bone marrow infiltration by malignant cells.

Leukoerythroblastosis, which refers to the presence of both immature white blood cells (WBCs) and nucleated RBCs in the peripheral blood, is frequently seen on the peripheral blood smear along with teardrop RBCs (Fig. 15-4). Although leukoerythroblastosis can also be seen in reactive and congenital conditions, it is the result of malignant disease processes in approximately 63% of cases.³⁷ Leukoerythroblastosis with dacryocytes or teardrop-shaped RBCs is often a clue to

TABLE 15-2 Endocrine Disorders: Type of Anemia, Pathogenesis, and Treatment

Endocrine Disorder	Type of Anemia	Pathogenesis (Cause of)	Treatment
Hypopituitarism	N/N	Loss of trophic function of pituitary hormones	Exogenous replacement of thyroid hormone Androgens and/or estrogens Administration of corticosteroids
Adrenal insufficiency	N/N	Idiopathic (unknown cause) In Addison disease – autoimmune	Exogenous source of corticosteroids
Hypothyroidism	Mild macrocytic (Macronormoblastic)	Hypothyroidism	Exogenous thyroid hormone
Hyperparathyroidism	N/N	Increased parathyroid hormone	Parathyroidectomy or medical treatment
Hypogonadism	N/N	Defective secretion of/from the gonads	Androgens
Diabetes – Type 1	N/N to mild macrocytic	Autoimmune (gastritis) Decreased absorption of Iron or B ₁₂	Iron or B ₁₂
Diabetes – Type 2	N/N	Inflammation Iron deficiency EPO deficiency Decreased RBC life span Decreased EPO response Therapy related	Glycemic control (treatment of underlying disease) Iron (if deficient)

N/N = normocytic/normochromic anemia.

BOX 15-3 Mechanisms of Myelophthitic Anemia**Direct Effects**

- Replacement of marrow by malignant cells
- Primary hematologic malignancy
- Ineffective erythroid production
- Qualitative reduction in erythropoiesis
- Metastatic marrow infiltration
- Quantitative reduction in erythropoiesis
- Replacement of marrow by fibrosis

Indirect Effects

- Anemia of malignant disease/anemia of chronic inflammation
- Anemia of associated organ failure (e.g., renal, hepatic)
- Malnutrition and vitamin deficiency
- Microangiopathic hemolytic anemia
- Immune hemolytic anemia

Treatment-Associated Anemia**Immediate**

- Chemotherapy
- Radiation therapy

Late

- Secondary myelodysplasia or leukemia
- Idiopathic
- Depleted marrow reserve
- Microangiopathic hemolytic anemia

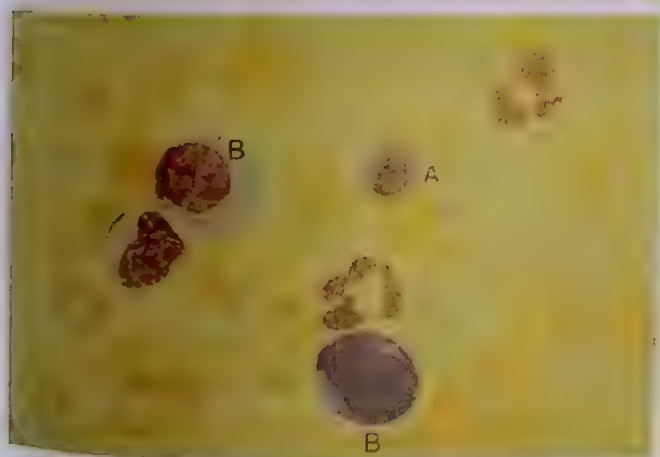


FIGURE 15-4 Leukoerythroblastosis, a peripheral blood picture that often accompanies marrow infiltration by tumors (myelophthitic anemia). Note the presence of immature (A) red and (B) white blood cells.

marrow infiltration by the tumor or by bone marrow fibrosis (Fig. 15-5) as opposed to a reactive process that usually demonstrates leukoerythroblastosis and normal RBC morphology (Fig. 15-6).

Clinical Findings

Most patients with myelophthitic anemia are already diagnosed with a systemic disease involving a malignancy (hematologic and nonhematologic) or fibrosis. In addition to the primary issue, patients will show evidence of pancytopenia due to the abnormal overcrowding of the bone marrow space.

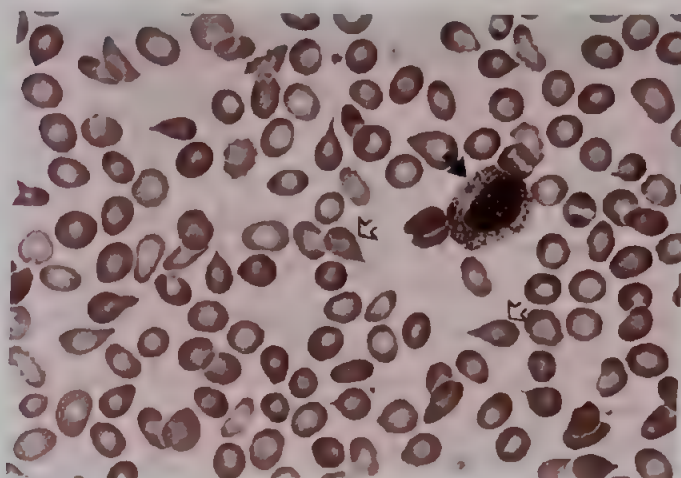


FIGURE 15-5 Peripheral blood smear from an individual with myelophthitic anemia. Note left-shifted granulocyte precursors (solid arrow). Teardrop-shaped red blood cells (open arrows) are indicative of marrow fibrosis in this type of leukoerythroblastic reaction. Nucleated red blood cell precursors were seen in other fields.

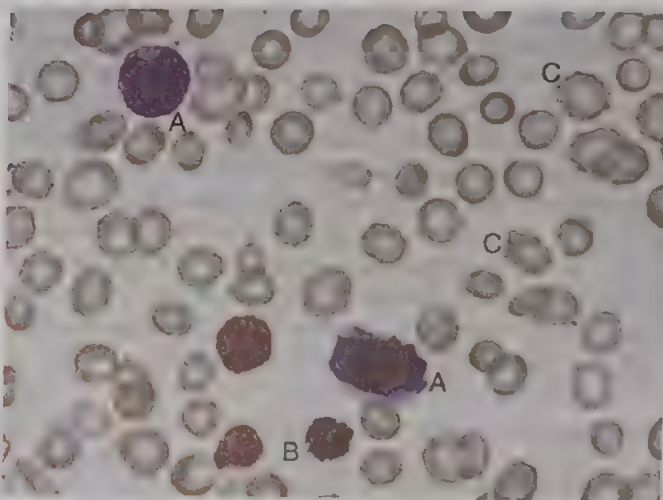


FIGURE 15-6 Peripheral blood smear from a neonate with leukoerythroblastosis caused by severe blood loss associated with birth (a reactive condition). (A) Left-shifted granulocyte precursors and (B) nucleated red blood cell precursors are seen. Although some (C) "burr" cells are seen, no teardrop-shaped red blood cells are identified.

Laboratory Evaluation

Myelophthitic anemia is usually classified as normocytic, normochromic, and hypoproliferative. As stated earlier, many other processes can occur to change the severity or morphology of the anemia. However, the most important change seen in myelophthitic anemia is leukoerythroblastosis with teardrop-shaped RBCs, indicating bone marrow replacement and/or bone marrow fibrosis and a poor prognosis because of abnormal involvement of hematopoietic bone marrow. Any polychromasia seen in this type of anemia is due to "crowding out" of normal red blood cell precursors from the bone marrow by tumor and not to the premature release or increased production of precursors from bone marrow, as would be seen in anemia with reticulocytosis; RBC morphology in myelophthitic anemia is outlined in Table 15-3.

TABLE 15-3 RBC Morphology in Myelophthytic Anemia

Morphology	Pathophysiology
Leukoerythroblastosis	Myelophthysis, marrow fibrosis
Teardrop RBC	Marrow fibrosis
Schistocytes*	Microangiopathic hemolytic anemia
Helmet cells*	Microangiopathic hemolytic anemia
Burr cells*	Microangiopathic hemolytic anemia
Hypochromia	Chronic blood loss, ↓ Fe

*Disseminated carcinomas; may be associated with disseminated intravascular coagulation. Fe = iron.

This crowding results in immature leukocytes and RBCs in the peripheral blood. Extensive marrow invasion/involvement can lead to pancytopenia. Essentially, the bone marrow space loses both its hematopoietic cellularity and its proper micro-environment, resulting in a hypoproliferative anemia.

Treatment

In severe cases of bone marrow replacement, transfusions are needed to correct the patient's anemia. Chemotherapy or radiation therapy protocols are often used to reduce the burden of malignancy involving the bone marrow while at the same time worsening anemia in the short term. Eradication of malignancy from the bone marrow offers the only chance for reconstitution of the marrow by hematopoietic precursors and endogenous correction of the anemia. Additional treatment may also include the correction of nutritional deficiencies (folate, B₁₂), iron therapy, transfusion, and/or EPO-stimulating agents if indicated (palliative care or kidney disease).³⁶

Anemia Associated With Viral Infections

Anemia has been associated with many different viral infections and often presents as normocytic, normochromic, hypoproliferative anemias. Parvovirus B19 has been shown to cause chronic anemia and pure red cell aplasia (see Chapter 13, Normocytic Normochromic Anemias). Epstein-Barr virus, the causative agent of infectious mononucleosis, has been associated with rare cases of hemolytic anemia. Hepatitis infections have been shown to contribute to the anemia of liver disease. While often present, the anemia is not typically a diagnostic feature of the viral infection.

SARS-CoV-2 and COVID-19

Etiology and Pathophysiology

SARS-CoV-2 was first recognized in December of 2019. Although the terms are often used interchangeably, SARS-CoV-2 refers to a novel strain of coronavirus that causes the infectious disease known as COVID-19.

Several mechanisms have been proposed for the effect of SARS-CoV-2 infection on hematopoiesis including inflammation, iron dysregulation, hemoglobin dysfunction, and direct infection of erythroid precursors.³⁸⁻⁴⁰ The pathophysiology of anemia in viral infections is outlined in Box 15-4.

While anemia has not been a diagnostic criterion for COVID-19, a declining trend in hemoglobin can be seen in

BOX 15-4 Pathophysiology of Anemia in Viral Infections

SARS-CoV-2 and COVID-19

- Inflammation
 - Cytokine Storm (IL1, IL6, TNF α , IFN γ)
 - Inhibition of erythropoiesis
 - Increased hepcidin production
- Iron dysregulation
 - Decreased serum iron
 - Hyperferritinemia
 - Hepcidin "mimicry" by SARS-CoV-2
- Hemoglobin dysfunction
 - Interaction of SARS-CoV-2 and the hemoglobin molecule
- Direct infection of erythroid precursors
 - Inhibition of erythropoiesis

HIV and AIDS

- Suppression of erythropoiesis caused by cytokines IL-1 and TNF- α
- Phagocytosis of erythroblastic cells by bone marrow histiocytes
- Antibodies to HIV, which circulate in bone marrow and destroy red cell precursors
- Production of complement protein, C3, and IgG, which form immune complexes on red cell membranes, causing hemolysis
- Infections of red cell precursors by parvovirus B19 and *Mycobacterium*, halting the maturation process in the bone marrow (aplastic anemia)
- Inhibitory serum protein, which results in a decrease in BFU-E
- Defective EPO response to anemia
- Treatment with AZT, which inhibits DNA synthesis of blood cells and results in a macrocytic anemia

severe cases. This is most likely due to the long life span of the RBC and the time necessary to reflect a decrease in RBC production. Inflammatory cytokines (IL1, IL6, INF- γ , and TNF- α) associated with the cytokine storm (typical of severe SARS-CoV-2 infections) have been shown to interfere with erythropoiesis.³⁸ Patients with more severe and prolonged cases of COVID-19 have hemoglobin levels that decreased more significantly compared with those who had milder cases.³⁸

Iron dysregulation has been seen in SARS-CoV-2 infections, including decreased iron levels and increased ferritin and hepcidin levels. Both ferritin and hepcidin are acute phase reactants that have been shown to increase in viral infections and play a role in cytokine storm formation.⁴⁰ Increased ferritin levels have been reported in patients with COVID-19, and hyperferritinemia has been associated with worse outcomes.^{40,41} Hepcidin is increased by the inflammatory effects of the cytokine storm (IL-6) as seen with ACI. Additionally, SARS-CoV-2 is thought to mimic the function of hepcidin, increasing the cellular storage of iron (hepatocytes, enterocytes, and macrophages).^{39,41} This iron dysregulation would ultimately lead to iron-restricted erythropoiesis and anemia while having an iron toxicity profile in the tissue due to hyperferritinemia. This increased tissue iron leads to ferroptosis (iron-mediated oxidative stress induced apoptosis of the cell). Although the cause of olfactory and gustatory dysfunction in SARS-CoV-2

remains unknown and may have many contributing factors, ferroptosis and decreased serum iron have been linked to cognitive disturbances, including the loss of taste (ageusia) and smell (anosmia) commonly associated as symptoms of COVID-19.^{39,41-43} It has been postulated that hyperferritinemia is the link to severity of COVID and risk of mortality.³⁹⁻⁴¹

Another potential mechanism of anemia in SARS-CoV-2 infection is direct viral infection of the erythrocytes and progenitors. This would allow for both interaction with hemoglobin and direct inhibition of erythropoiesis. Entry of the virus through CD147 allows interaction of the viral ORF8 protein with the porphyrin ring in hemoglobin (a potential mechanism previously described in studies with *Plasmodium spp.*) and has led to the consideration of a possible “acute acquired porphyria” in COVID-19.³⁹ Increased levels of lactate dehydrogenase (LDH) are seen in severe cases pointing to the possibility of increased hemolysis; however, LDH is also elevated with tissue damage, which could be a result of lung damage or inflammation seen in severe cases.^{39,41} The direct viral infection in erythrocyte precursors may also limit or inhibit erythropoiesis.

Clinical Findings

COVID-19 has been shown to have a full range of severity from asymptomatic, flu-like illness, pneumonia, acute respiratory distress, and even multiorgan failure with viral sepsis.³⁸⁻⁴⁰ Common early symptoms of SARS-CoV-2 infection include olfactory and gustatory dysfunction in up to 86% of those infected.^{41,42} Severity of disease is thought to be complicated by preexisting conditions such as diabetes and cardiovascular disease. Additionally, patients with preexisting conditions that have been linked to more severe cases of COVID may be anemic before infection.

Laboratory Evaluation

SARS-CoV-2 infections have presented with a variety of hematological abnormalities including lymphopenia, neutrophilia, and thrombocytopenia.³⁸⁻⁴¹ Hypercoagulability, increased D-dimer, and prolonged prothrombin time have also been observed in severe cases of COVID-19.^{38,41} Long-term studies of the effect of infection on hematopoiesis have not been completed at this time.

Children infected with SARS-CoV-2 typically have normal hemoglobin levels and fewer thrombotic complications even with severe infection. The exception to this is when the child presents with multisystem inflammatory syndrome where anemia is a common feature along with lymphocytopenia, neutrophilia, thrombocytopenia, hyperferritinemia, and high levels of inflammation.⁴⁴

HIV and AIDS

Similar to the relationship of SARS-CoV-2 and COVID-19, human immunodeficiency virus (HIV) refers to a novel viral strain that causes the infectious disease known as acquired immunodeficiency syndrome (AIDS).

HIV infection is a devastating condition caused by direct destruction of CD4 T lymphocytes (helper-inducer cells) by HIV. By reducing the CD4 T-cell count, HIV renders the host immunocompromised and susceptible to many opportunistic pathogens. Most HIV-infected patients develop one or more

cytopenias during the course of the disease. Anemia develops early and becomes more severe as the disease progresses. The anemia associated with HIV infection can be worsened by concurrent conditions, such as one or more opportunistic infections. Anemia is the most common cytopenia associated with HIV infection (in up to 70% of patients).⁴⁵ Decreased production of RBCs can be attributed to several possible mechanisms including therapeutic interventions, inflammation, bone marrow suppression from direct infection of erythrocyte precursors by HIV, and immune response to HIV.⁴⁵ For the most part, the anemia of HIV infection and AIDS is a subset of ACI. HIV/AIDS treatment protocols have also been associated with a side effect of anemia.

Anemia of Prematurity

Etiology and Pathophysiology

Infants are typically born with HGB values of 17.0 g/dL or higher (reference range of 14.0 to 22.0 g/dL). During the first month of life, all infants experience a decrease in their circulating RBC mass. The concentration of HGB declines rapidly to the lowest value, approaching 9.0 g/dL within the first 2 months.⁴⁶⁻⁴⁸ This decline in red blood cell mass is due to many factors, including the lack of production of EPO, decreased life span of the newborn's RBCs, hemoglobin switch from HgbF to HgbA, and availability of iron.⁴⁷

Newborns have been shown to have a decline in erythropoiesis and little detectable EPO in the first few months of life as the site of production of EPO switches from the liver to the kidney.^{46,47} This switch is not complete until about 1 to 2 months after birth. Newborns “tolerate” hypoxia and have an inadequate compensatory response (such as hyperventilation and increased EPO production). There is also a sex-dependent response in EPO production as it is regulated by the sex hormones: testosterone (increases production) and estradiol (decreases production). Interestingly, female newborns tolerate the anemia better than male newborns.⁴⁹ The central nervous system has been shown to produce and use EPO to modulate the response to hypoxia, and these mechanisms are not fully developed at birth adding to the “tolerance” of hypoxia.⁴⁹ Lungs become the source of oxygen rather than the placenta, increasing the arterial oxygen saturation. Additionally, HgbF has a higher oxygen affinity than HgbA (see Chapter 2, The Red Blood Cell). As more HgbA is produced (and 2,3 diphosphoglycerate), there is a shift to the right on the oxygenation dissociation curve, increasing the delivery of oxygen to the tissues that would ultimately decrease the production of EPO as tissue oxygenation is improved after birth.⁴⁶ The EPO production increases as the HGB reaches the nadir value of 9.0 g/dL, and therefore erythropoiesis increases.

Varying levels of anemia are due to the many physiological factors that are present during the first 1 to 2 months of life (Box 15-5). The anemia is usually well tolerated in full-term (39 weeks) infants. In the past, this “physiological” state was termed anemia of infancy, but the term is no longer recognized because it occurs in the absence of any recognized nutritional deficiency and is characteristic of even the healthiest of infants. Screening for anemia in infants is recommended at 1 year of age by the American Academy of Pediatrics and

BOX 15-5 Contributing Factors in the Anemia of Prematurity

- Rapid growth
- EPO production by the neonatal liver (switch to kidney ~1 to 2 months)
- Increased rate of clearance of EPO from neonatal plasma
- Increased plasma volume/Increased volume of distribution of EPO
- Short half-life of neonatal red blood cells
- Hemoglobin switch from HgbF to HgbA (oxygen dissociation curve)
- Nutritional deficiencies
- Chronic blood loss or "Lab draw" anemia (usually seen in hospitalized premature neonates)

the World Health Organization, although selective screening in infants with additional risk factors (low birth weight, feeding issues, poor growth) or hereditary risks should also be performed, as infants with mild anemia may not be symptomatic.⁴⁸

The anemia of prematurity involves the same alterations in the production, plasma distribution, and clearance of EPO. Unlike full-term neonates, preterm infants demonstrate earlier and more severe declines in HGB values despite increased reticulocytes.^{46,47} An additional complication of prematurity is an immature respiratory system that exacerbates the hypoxia. While the mainstay of the anemia in prematurity is the lack of EPO production, iatrogenic blood loss plays a significant role in the decrease in hemoglobin seen in premature infants under critical intensive care. To reduce the effect of iatrogenic blood loss, lab utilization measures can be taken to minimize the amount of blood drawn, including assessing the timing and clinical necessity for testing, using pediatric phlebotomy tubes to reduce total volume, and utilizing point of care testing methodologies that require less blood per test.⁵⁰

Clinical Findings

Patients present with various symptoms depending on the severity of anemia. Patients suffering from hypoxia can present with pale color, tachycardia (a rapid heartbeat), tachypnea (rapid breathing rate), apnea (interruption of breathing or irregularity of respiration), and bradycardia (slower than normal heart rate).

Laboratory Evaluation

The physiological anemia of infancy causes a decrease in RBC mass leading to a mild, normocytic, normochromic anemia with reticulocytopenia that corrects as the production of EPO increases. Other cell lines such as platelets and WBCs are not affected. In premature infants, this physiological anemia is exaggerated by an immature respiratory system and iatrogenic blood loss associated with frequent phlebotomy necessary for testing to support critical intensive care. The longer the stay and more critical the illness, the more prominent this blood loss becomes.

Treatment

Treatment of choice for low HGB values in children is nutritional supplementation, particularly iron and B₁₂/folate. If anemia causes symptoms such as hypoxia, packed RBC units are the product of choice for transfusion. However, in the absence of symptoms, no treatment is required for this physiological anemia of infancy as it usually corrects on its own. Iron and vitamin supplementation are often recommended. Where physiological anemia of infancy is well tolerated and rarely requires treatment, anemia associated with prematurity is not well tolerated and may require transfusion support. In addition to iron and vitamin supplementation, EPO-stimulating agents may be warranted depending on the symptomology.

Acknowledgment

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SUMMARY CHART

- Anemias produced by systemic diseases are perhaps the most common hematologic abnormalities encountered in the clinical laboratory; these are generally hypoproliferative anemias characterized by decreased or normal reticulocyte counts (inappropriately normal reticulocyte count).
- Chronic kidney disease (CKD) may be associated with anemia, abnormal platelet and white blood cell function, and coagulopathy.
- The major causes for the anemia of CKD are (1) decreased production of EPO by damaged kidneys, (2) suppression of erythropoiesis by the uremic toxins that accumulate in renal failure, and (3) slightly to moderately decreased RBC life span. Other causes of anemia may complicate the anemia of CKD.
- The anemia of CKD is hypoproliferative (normal to decreased reticulocyte count); it is normocytic and normochromic with the presence of burr cells.
- Chronic liver disease may show RBC morphology that includes macrocytosis, target cells, acanthocytes, and thrombocytopenia if hypersplenism is present; a microcytic, hypochromic anemia is evident in the presence of chronic blood loss with iron deficiency.
- The bone marrow in chronic liver disease shows normocytic to macrocytic maturation in RBC precursors. RBCs have decreased survival because of increased membrane lipids and lack of deformability (e.g., acanthocytes or "spur cell" anemia).
- Anemia caused by chronic liver disease may also involve alcoholism, which is the result of ethanol toxicity to precursor cells in the bone marrow. The bone marrow shows macrocytosis, vacuolization of erythroblasts, and abnormal hemoglobinization of erythroid series precursors. The peripheral blood shows macrocytosis and may show spur cells.

SUMMARY CHART—cont'd

- A dimorphic blood picture may be seen in patients with anemia of chronic liver disease caused by alcoholism because of iron deficiency caused by chronic blood loss; the MCV is normal and the RDW increased.
- Anemia associated with endocrine disease is usually normocytic and normochromic, and may be caused by diabetes, pituitary dysfunction, adrenal insufficiency, Addison disease, thyroid disease, hyperparathyroidism, hypogonadism, or a combination of endocrinopathies.
- Myelophthitic anemia is dependent on the type of infiltration, the patient's therapy, and the extent of bone marrow involvement.
- Myelophthisis refers to infiltration of the bone marrow by a malignant tumor; the most characteristic peripheral blood morphology associated with myelophthisis is leukoerythroblastosis (the presence of immature myeloid precursors and nucleated RBCs) with teardrop-shaped red cells (dacrocytes).
- Viral infections have been associated with anemia. Patients infected with SARS-CoV-2 may be anemic due to preexisting conditions, iron dysregulation (decreased serum iron, increased hepcidin, hyperferritinemia), increased inflammation (cytokine storm), hemoglobin dysfunction, and direct infection of erythroid precursors.
- Anemia associated with HIV and AIDS may be due to inflammation, decreased erythropoiesis, increased erythrophagocytosis, immune response to HIV, and side effects of treatment protocols.
- Anemia in infancy is physiological and due to decreased levels of EPO (to the switch from liver to renal EPO production), decreased RBC life span, hemoglobin switch from HgbF to HgbA, and iron availability.
- Anemia of prematurity is seen in premature infants and increased by iatrogenic blood loss anemia associated with the intensive care.

CASE STUDY 15-1

A 22-year-old woman was admitted from the emergency department with fever, dysuria, and lower back pain. Immediate laboratory results revealed the following:

Urinalysis	CBC
Urine reagent strip:	WBC: $11.8 \times 10^9/L$
4+ urine protein	RBC: $2.9 \times 10^{12}/L$
1+ hemoglobin	Hgb: 8.3 g/dL
Urine microscopic:	Hct: 25%
Many bacteria	MCV: 88 fL
Moderate blood	MCH: 29 pg
Moderate leukocytes	MCHC: 32%
Cast: few hyaline and few granular	RDW: 14.7% Platelets: $315 \times 10^9/L$
Blood Chemistry	1+ anisocytosis
BUN 113 mg/dL	1+ poikilocytosis
Creatinine 7.7 mg/dL	1+ burr cells

Blood cultures were drawn and eventually produced only a few colonies of coagulase-negative *Staphylococcus* (probably skin contaminants). Urine cultures grew $>100,000$ colony-forming units per milliliter of *E. coli*. Antibiotic treatment was begun. Blood was still present in the patient's urine after 2 days, and bleeding was noted from intravenous infusion sites. Coagulation tests were ordered before the patient was to have a kidney biopsy performed. These showed:

Platelet count: $296 \times 10^9/L$
 PT: 11.7 seconds (normal range: 10.0–12.9 seconds)
 APTT: 32 seconds (normal range: 23–35 seconds)
 PFA: prolonged with EPI and ADP cartridges

(PT is prothrombin time; APTT is activated partial thromboplastin time; PFA is platelet function assay)

This patient was suffering from renal failure and a urinary tract infection (UTI). The admitting CBC demonstrated a moderate anemia with some burr cells typical of renal disease. The anemia could be the product of several mechanisms. It was most likely a result of the anemia of chronic kidney disease. This may have been complicated by blood loss due to abnormal platelet function related to her renal failure.

QUESTIONS

1. Classify the anemia with regard to RBC indices.
2. List the laboratory parameters given in this case that coincide with anemia of chronic kidney disease.
3. Why would chronic iron loss be a concern in this patient?

ANSWERS

1. Normocytic, Normochromic
2. In the blood chemistry, the tests of kidney function: Blood urea nitrogen (BUN) and Creatinine are both elevated; casts in the urinalysis indicate kidney damage; disease, burr cells on peripheral smear correlate with kidney disease and uremia (raised level of urea and nitrogen waste in the blood)
3. Bleeding due to abnormal platelet function (note of the bleeding from IV sites and blood in urine) or loss of blood during dialysis can lead to chronic iron loss for patients with CKD. Decreased levels of EPO can also decrease intestinal absorption of iron through upregulation of hepcidin (see Chapter 7). Treatment for anemia of CKD would be to give EPO, but that is only effective if sufficient iron stores are present.

CASE STUDY 15-2

A 55-year-old man with a self-reported history of drinking 5 to 6 alcoholic beverages a day for several decades presents for an annual exam. He was noted to have a distended abdomen, yellow-tinged sclera, and slightly slurred speech. His laboratory testing revealed increased liver enzymes levels (alanine transaminase-ALT, aspartate transaminase-AST) and an elevated total bilirubin.

WBC: $10.0 \times 10^9/L$

RBC: $3.1 \times 10^{12}/L$

Hgb: 9.8 g/dL

Hct: 32%

MCV: 100 fL

MCH: 32 pg

MCHC: 31%

RDW: 15.5%

Platelets: $100 \times 10^9/L$

1 + anisocytosis

2 + poikilocytosis

1 + target cells

1 + acanthocytes (spur cells)

QUESTIONS

1. What would be the most likely cause of this patient's anemia?
2. What would you expect the hemostasis testing results to reveal?
3. How are the poikilocytes formed in this condition?

ANSWERS

1. Chronic liver disease caused by excessive alcohol consumption
2. Hemostasis testing (PT – prothrombin time & APTT – activated partial thromboplastin time) is prolonged in patients with liver disease due to the decreased production of coagulation factors, particularly the vitamin K dependent factors: II, VII, IX, X.
3. Abnormal lipid metabolism in patients with liver disease causes increased lipid in the RBC membrane leading to macrocytes, acanthocytes, and target cell formation.

REVIEW QUESTIONS

1. What is the primary cause of anemia associated with renal disorders and renal failure?
 - a. Blood loss from dialysis
 - b. Decreased EPO production
 - c. Hemolytic processes
 - d. Vitamin B₁₂ and folate deficiencies
2. Patients with anemia of chronic renal disease are usually treated with:
 - a. Chemotherapy
 - b. Radiation
 - c. EPO
 - d. Vitamin B₁₂
3. What is a typical appearance of anemia associated with liver disease?
 - a. Hyperchromic with spherocytic red blood cell morphology
 - b. Hypochromic, microcytic red blood cell morphology
 - c. Macrocytic, megaloblastic red blood cell morphology
 - d. Macrocytic, normoblastic red blood cell morphology
4. Macrocytosis in liver disease is caused by all of the following except:
 - a. Abnormal lipid metabolism
 - b. Direct effects of alcohol
 - c. Iron deficiency
 - d. Vitamin B₁₂ and folate deficiency
5. Alcoholics are at increased risk of acquiring which disorder?
 - a. Sideroblastic anemia
 - b. Anemia of chronic kidney disease
 - c. Anemia of infection
 - d. Anemia of endocrine dysfunction
6. What are the typical hematologic findings associated with anemia from endocrine dysfunction?
 - a. Anemia, abnormal platelets, and coagulopathy
 - b. Marked anisocytosis and poikilocytosis, and dysfunctional leukocytes
 - c. Mild normocytic, normochromic anemia
 - d. Target cells, macrocytes, and acanthocytes
7. Leukoerythroblastosis with teardrop-shaped red blood cells is indicative of:
 - a. Abnormal lipid metabolism
 - b. Bone marrow fibrosis
 - c. Bone marrow stress
 - d. Renal disease
8. The anemia of prematurity is a subset of anemia of infancy that is:
 - a. Not enhanced by an immature respiratory system
 - b. Often complicated by blood loss/frequent blood drawing
 - c. Unnecessary to treat as it is self-resolving
 - d. Well tolerated

See answers at the back of this book

CHAPTER 16

Benign White Blood Cell Disorders

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CHAPTER OUTLINE

Neutrophils
Neutrophil Function
Disorders of Neutrophils
Eosinophils
Basophils

Monocytes
Lymphocytes
Absolute Lymphocytosis: Reactive
versus Malignant Causes
Lymphocytopenia

Summary Chart
Case Study 16-1
Case Study 16-2
Review Questions

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 16-1 Characterize the changes in neutrophil count and morphology that develop in response to bacterial infection.
- 16-2 Define the three modes of neutrophilic migration.
- 16-3 Describe the changes in migration pattern and appearance that occur in a neutrophil as the result of chemoattractant stimulation.
- 16-4 Explain the contents and functions of primary, secondary, and tertiary neutrophilic granules.
- 16-5 Contrast oxygen-dependent and independent phagocytosis.
- 16-6 Define absolute and relative leukocytosis and leukopenia.
- 16-7 Appraise the etiology and classic clinical features of Chédiak-Higashi syndrome, May Hegglin anomaly, Alder-Reilly anomaly, and Pelger-Huët anomaly including the associated changes in neutrophil morphology.
- 16-8 Compare and contrast changes in morphology related to white blood cell anomalies.
- 16-9 List several disorders that present with lymphocytosis.
- 16-10 Differentiate morphological features of infectious mononucleosis and other reactive lymphocytoses.
- 16-11 List clinical features of infectious mononucleosis.
- 16-12 Evaluate how the Epstein-Barr virus affects the B- and T-lymphocyte populations.
- 16-13 Utilize laboratory results for distinguishing infectious mononucleosis from other lymphocytoses.
- 16-14 Analyze the molecular basis for the inherited white blood cell disorders.

This chapter focuses on the function of white blood cells, or leukocytes. Leukocytes can be divided into two groups: granulocytes and lymphocytes (see Chapter 1 for maturation process). Granulocytes generate from the myeloid stem cell and can be divided into four subsets of cells: neutrophils, eosinophils, monocytes, and basophils. These cells are differentiated based on cellular morphology (by light and electron microscopy) and on the staining characteristics and contents of cytoplasmic granules. Lymphocytes generate from a lymphoid stem cell that have distinctive cellular morphology, and serve to benefit the body with the production of antibodies.

As you will learn in this chapter, leukocytes play an important role in the body's immune response, and disorders influencing these cells can have a significant effect on the health of patients.

Neutrophils

Neutrophils are the most numerous leukocytes found in the peripheral blood, accounting for 50% to 70% of all circulating white blood cells (WBCs) in the adult. Similar to monocytes, neutrophils function as phagocytes that are capable of amoeboid movement into the tissues to engulf and destroy bacteria or fungi; they are the first phagocytic cells to mobilize to a site

of infection. Neutrophils also play a role in mediating inflammatory processes.

Mature neutrophils (also called segmented neutrophils or polymorphonuclear leukocytes) are easily recognized on Wright's-stained peripheral blood smears by their dense multilobed nucleus and pinkish-tan cytoplasm, evenly dispersed with pinkish-purple granules. Three major types of cytoplasmic granules are present in the mature neutrophil. Primary (azurophilic or nonspecific) and secondary (specific) are visible with light microscopy. A third type of granule (tertiary) has been identified using electron microscopy. Cytoplasmic granules are present at various stages of development and contain enzymes, most of which are involved in the killing and digestion of bacteria and fungi (Box 16-1). The cytoplasm of mature neutrophils also contains ficolin-1 rich granules and secretory vesicles.¹

Mature neutrophils are smaller than myeloid precursors and thus are more mobile and deformable. After exiting the proliferative pool of the marrow and entering the bloodstream, mature neutrophils divide equally into marginating and circulating pools, between which there is a constant exchange of cells. The marginating pool, not measured by peripheral blood sampling, consists of cells adhering to vessel endothelium within the vascular spaces. Marginating cells can either return the circulating pool in response to epinephrine or enter the tissues by diapedesis in response to inflammation or injury. Diapedesis is a unidirectional process and mediated by selectins, chemokines, and integrins.^{2,3} Neutrophils in the circulating pool leave the blood by random migration after a half-life of approximately 7 hours and do not return to the bloodstream from tissues. Little is known

of the kinetics of neutrophils after having entered the tissues; they are believed to remain in tissues for several days, where, if not used in an inflammatory process, they die by apoptosis or are destroyed by other phagocytic cells of the reticuloendothelial system or marrow.⁴ The bone marrow replaces the neutrophils that have exited the bloodstream with cells from the bone marrow storage pool. The daily production of neutrophils is approximately $1 \times 10^{11}/L$ in adults, with 20% remaining in circulation.

Variations in the number and morphology of leukocytes in the bloodstream and bone marrow have long been used as clinical guides for the diagnosis of many diseases. These variations reflect the response of normal leukocytes to an underlying disease or indicate a primary disorder intrinsic to leukocytes, such as leukemia. A thorough knowledge of established reference values and morphological characteristics of all cellular elements is important for recognizing and interpreting unexpected findings.

CRITICAL THINKING QUESTION

16-1 If a child is crying while their blood is drawn for a CBC, why will the WBC result be high?

See answers to all Critical Thinking Questions at the back of this book.

Neutrophil Function

Subpopulations of neutrophils with improved immune regulation, enhanced tumor killing, and tissue repair have been recently identified; however, the origin and development of these specialized phenotypes is still unknown.^{5,6} Despite the apparent heterogeneity in this cell population, the main function of the neutrophil is the internalization of microorganisms for destruction, a process referred to as phagocytosis. Once bacteria infiltrate the tissues, neutrophils are stimulated for immediate action. For the purposes of discussion, phagocytosis is described as occurring in three distinct phases: migration and diapedesis; opsonization and recognition; and ingestion, killing, and digestion (Fig. 16-1).

BOX 16-1 Contents of Neutrophil Granules

Primary/Nonspecific or Azurophilic Granules

- Myeloperoxidase
- Lysozyme
- α -Defensin
- Bactericidal/permeability increasing protein
- Serine Proteases (Proteinase 3, Cathepsin G, Elastase, Azurocidin, Neutrophil serine protease 4)
- Acid hydrolases (Cathepsin B/G/D, β -Glucuronidase, β -Glycerophosphatase, α -Mannosidase)

Secondary/Specific Granules

- Lactoferrin
- Lysozyme
- Gelatinase
- Collagenase
- Histaminase
- Heparinase
- Neutrophil Gelatinase associated lipocalin (NGAL)
- Signal regulatory protein alpha (SIRP- α)
- Transcobalamin I and III
- Plasminogen activator

Tertiary Granules

- Gelatinase
- Lysozyme
- Alkaline phosphatase

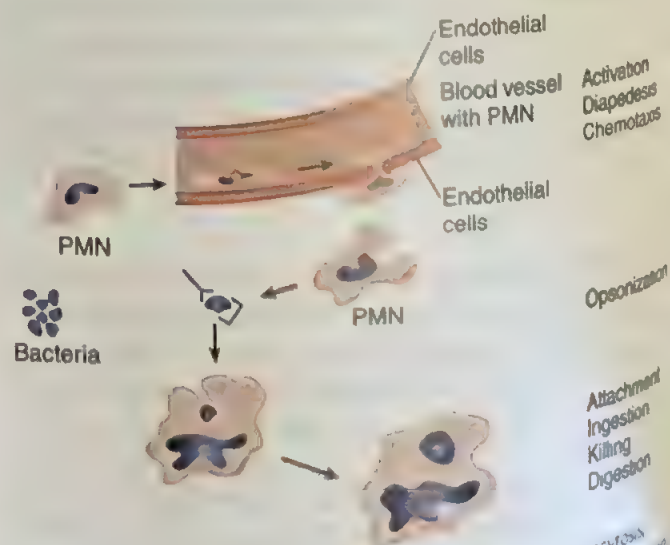


FIGURE 16-1 Illustration of the phases of neutrophilic phagocytosis, which include activation, diapedesis, chemotaxis, opsonization, ingestion, killing, and digestion. PMN = polymorphonuclear neutrophil. (From Abramson JS, Wheeler JG. *The Natural Immune System*. Oxford: Oxford University Press; 1993, p 110, with permission.)

Migration and Diapedesis

Neutrophils in the marginating pool roll along vessel endothelium in a random and nondirectional pattern called **locomotion**, until a site of injury or infection is encountered. Bacteria and sites of inflammation in the host send out signals in the form of chemoattractants (Table 16-1), which stimulate changes in morphology and migration pattern.^{2,4} The chemoattractant forms a concentration gradient, whereby the neutrophil migrates toward the area of highest concentration. A reengineering of the plasma cell membrane occurs that transforms the neutrophilic shape from smooth and round to ruffled and flat, with pseudopods and a tail (Fig. 16-2). Under the stimulus of chemoattractants, the activated neutrophil migrates in a repetitive, wavelike motion, crawling along the endothelium toward the site of infection. The process of directional migration under the guidance of chemoattractants is known as **chemotaxis**. The neutrophil adheres to endothelial receptors and, by diapedesis, penetrates through narrow junctions between endothelial cells into the tissues. In diapedesis, the neutrophil is briefly retained by the vascular basement membrane but then enters the tissues by passing through small openings in this membrane. Chemoattractants further accelerate the rate of neutrophil migration by a process known as **chemokinesis**.⁷ With neutrophils being the first phagocytes to migrate to the site of infection, the lag time between microorganism invasion and timely neutrophilic migration is crucial for limiting the spread of (or even preventing) infection. All three modes of neutrophilic migration contribute to the efficient mobilization of neutrophils to the site of injury (Table 16-2).

Recognition and Opsonization

Neutrophils have membrane receptors that aid in the direct or indirect recognition and attachment to pathogens.⁸ Circulating

TABLE 16-2 Three Modes of Neutrophil Migration

Mode of Migration	Characteristics
Locomotion (random)	Nondirectional
Chemokinesis	Nondirectional acceleration of migration speed
Chemotaxis	Directional

immunoglobulin and activated complement components act as opsonins, coating the surface of the bacteria and permitting recognition and ingestion by the neutrophil. Membrane-bound immunoglobulin is necessary for optimal phagocytosis. The plasma membrane of the neutrophil carries receptors for the FC fragment of immunoglobulin G (IgG) molecules and activated complement. Proteins that most effectively mark bacteria for recognition and attachment are IgG1, IgG3, C3b, and C3bi.⁹ Nonopsonic pattern recognition receptors directly recognize pathogen-associated molecular patterns on the surface of microorganisms.^{4,8}

Intracellular Killing: Phagocytosis

The ingestion of the opsonized microbe begins as soon as the membrane surface receptor of the neutrophil and microbe bind together. Membrane pseudopods extend around and envelop the microbe, forming an isolated vacuole within the neutrophil cytoplasm known as a phagosome. Simultaneously, cytoplasmic granules and lysosomes migrate to and fuse with the membrane of the phagosome, forming a phagolysosome (Fig. 16-3). Once fusion is complete, the cytoplasmic granules undergo degranulation, whereby their contents are released into the phagolysosome. Studies suggest an orderly sequence of events for phagolysosome formation, with the release of lysosomes preceding the stepwise degranulation of tertiary, secondary, and finally primary granules. The ingested organism is exposed to the lytic activity of

TABLE 16-1 Chemical Factors That Signal Neutrophil Activation

Chemoattractants	Source
N-formyl oligopeptides	Bacteria
C5a, C3b, and C3bi factors	Complement
Interleukin-8	Monocyte
Leukotriene B ₄	Membrane phospholipid
Platelet-activating factor	Endothelium



FIGURE 16-2 The shape change of the neutrophil, from smooth and round to ruffled and flat with pseudopods, is a result of stimulation by chemoattractants. (Adapted from Kaplan S, Brams CA. Testing for Neutrophil Function. Advance. 1995; 7[1]:8, with permission.)

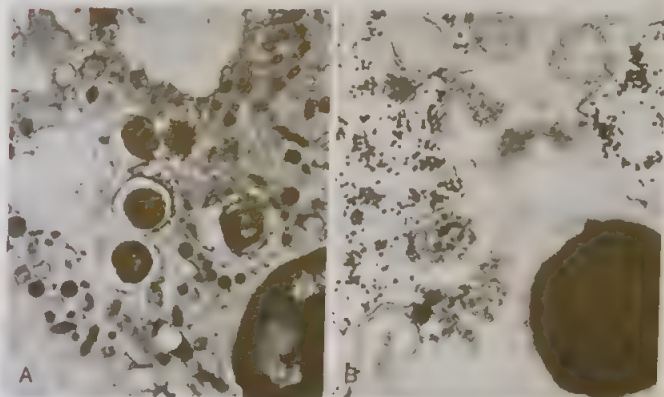


FIGURE 16-3 Electron microscopy of phagolysosome formation. (A) Staphylococci lie within phagocytic vesicles limited by sacs formed from inverted pieces of the neutrophil membrane. Cytoplasmic granules are approaching the phagocytic vesicles. (B) Higher magnification shows degranulation with the discharge of granule contents into the vicinity of the staphylococcus

granular enzymes within the phagolysosome, which leads to eventual killing and digestion.¹

Microorganism destruction (killing) is accomplished by oxygen-dependent and nonoxygen-dependent mechanisms. The nonoxygen-dependent mode of killing the internalized particle is represented by an alteration in pH and the release of the lysosomal and proteolytic enzymes into the phagolysosome.¹⁰ The lytic enzymes possess bactericidal activity that cleaves segments of the bacterial cell wall. Alternatively, oxygen-dependent killing involves the release of nicotinamide adenine dinucleotide phosphatase (NADPH) oxidase, which mediates the production of the active oxygen metabolites superoxide and hydrogen peroxide during a process known as the respiratory burst.⁸ These active oxygen metabolites are capable of microorganism injury. To enhance the antibacterial activity further, superoxide and hydrogen peroxide react with other preformed products present in primary, secondary, and tertiary neutrophilic granules that fuse with the phagolysosome to produce highly toxic agents such as reactive oxygen species and hypochlorous acid.¹⁰

Extracellular Killing

Neutrophils also mediate the extracellular killing of pathogenic microorganisms through the formation of neutrophil extracellular traps. In response to inflammatory cytokines or direct recognition of pathogen associated molecular patterns, neutrophils form extracellular antimicrobial webs, or “traps,” from cytosolic granules and nuclear chromatin.^{1,11} While productive to combat infection, upregulated release of neutrophil extracellular traps can also contribute to pathogenesis from autoimmunity and inflammation.¹²

Disorders of Neutrophils

Disorders of the neutrophilic cell line may predispose an individual to recurrent bacterial infections that are resistant to treatment. Neutrophilic disorders are classified as quantitative and qualitative, reflecting changes in neutrophil number and function, respectively. Quantitative disorders constitute a significant change in the absolute neutrophil number and are referred to as neutropenia (decreased) or neutrophilia (increased). Neutropenia limits the potential for effective defense against microbial invasion, increasing the risk of bacterial and fungal infection, with the level of risk proportional to the cause and duration of decrease.^{13,14} Alternatively, qualitative disorders are marked by neutrophil dysfunction as a result of impaired migration or altered bactericidal activity. Generally, any combination of deficiency or dysfunction in activation, migration, and diapedesis toward a pathogen or the recognition, ingestion, and killing of an opsonized particle can modify neutrophil function.^{8,15} Cytoplasmic or nuclear abnormalities also exist that affect the appearance of the cell but do not affect function.

Quantitative Disorders

Neutrophilia The classic response to infectious and inflammatory processes is an increase in the absolute or relative number of neutrophils, termed **neutrophilia**. In adults, neutrophilia is defined by absolute neutrophil values that exceed $7.7 \times 10^9/L$.¹⁶ Absolute counts are obtained by multiplying the

total WBC count by the percentage of neutrophils (and bands seen in the differential cell count). A low neutrophil count is not the sole indicator of disease and should be correlated with patient history as well as clinical and laboratory findings. The normal level of circulating neutrophils varies with age and race, and refers to mature segmented/polymorphonuclear and band forms, only.

An increase of neutrophils in response to inflammation or stress is called a **leukemoid reaction**. The accelerated release of neutrophils from the bone marrow reserve can be accompanied by a “shift to the left” that is defined as an increased number of metamyelocytes and band forms in the circulating pool.^{1,17} An increase in circulating neutrophils and immaturity is similarly observed in the early stages of neoplastic conditions, such as chronic myeloid leukemia (CML) and other chronic myeloproliferative disorders (see Chapter 18). The major distinction is that the myeloid precursors released during a leukemoid reaction, or infectious response, are more limited to the metamyelocyte and band stages; whereas, in neoplastic processes, earlier precursor cells such as myelocytes, promyelocytes, and blasts are also present. Further, a marked increase in total leukocyte values, defined as **leukocytosis** (total leukocyte counts exceeding $>50 \times 10^9/L$) or **hyperleukocytosis** (counts exceeding $>100 \times 10^9/L$) are more often present in malignant conditions, while reactive causes of leukocytosis do not typically exceed this value.^{17,18} Box 16-2 lists the causes of reactive leukocytosis and neutrophilia. Leukocyte alkaline phosphatase (LAP) cytochemical staining is one way to differentiate neutrophilic response to infection (leukemoid reaction) from chronic myeloid leukemia, where the LAP is increased in leukemoid reaction and

BOX 16-2 Causes of Secondary Reactive Neutrophilia (Absolute Count $> 6,000/mm^3$ or $6.0 \times 10^9/L$)

- **Infections**
 - Bacterial (most common)
 - Toxoplasma, mycobacterium, leptospiral, and viral (less common)
- **Inflammatory Disorders**
- **Tissue Necrosis**
 - Burns
 - Trauma
 - Infarct
 - Acute gout
- **Metabolic**
 - Uremia
 - Ketoacidosis
 - Eclampsia
- **Other Causes Unrelated to Infection or Inflammatory Process**
 - Stress
 - Rigorous exercise
 - Smoking
 - Pregnancy
 - Trauma
 - Acute hemorrhage or hemolysis
 - Postsplenectomy

decreased in CML. In contrast to malignant causes, persistent idiopathic neutrophilia is a relatively common finding in adult patients and must be differentiated from chronic neutrophilic leukemia¹⁶ (see Chapter 18).

The kinetics of circulating neutrophils varies greatly depending on the type, duration, and intensity of the infection. The immediate response to infection is transient neutropenia, resulting from increased margination and accelerated delivery of neutrophils to the infected site. Within an hour, neutrophils are released from the bone marrow reserve into the bloodstream.¹ In the early phases of infection, the circulating half-life of neutrophils is shortened, metabolic changes occur in the microenvironment, and cell turnover is accelerated. Later, the circulating half-life returns to normal.³

In addition to changes in neutrophil number, reactive changes in neutrophilic morphology may be observed in leukemoid reaction. Leukemoid reaction is characterized by the presence of toxic granulation, Döhle bodies, and cytoplasmic vacuolization in the neutrophils (Figs. 16-4, 16-5, and 16-6). Toxic granulation is frequently associated with severe infection in which the cytoplasmic granules enlarge and take on darker staining properties than normal. The toxic granules



FIGURE 16-4 Toxic granulation (peripheral blood). Note the prominent dark-staining granules.



FIGURE 16-5 Döhle bodies (arrows). Note the large bluish bodies in the periphery of the cytoplasm.



FIGURE 16-6 Vacuolated neutrophils suggesting the presence of infection or a severe inflammation.

have been identified as peroxidase-positive primary granules. Toxic granulation may also be accompanied by the presence of Döhle bodies, pale blue inclusions at the periphery of the cytoplasm composed of aggregated strands of rough endoplasmic reticulum. Döhle bodies are similar in appearance to the inclusions found in the May-Hegglin anomaly (a hereditary leukocyte and platelet disorder, discussed later in this chapter). Lastly, in response to infection, the cytoplasm may become vacuolated and, occasionally, contain ingested microorganisms. All three of these morphological features are commonly observed, with toxic granulation being reported as the most common morphological change in response to bacterial infection, followed by Döhle bodies and cytoplasmic vacuolization.^{18,19} The presence of one or more of these reactive changes, in addition to other inflammatory markers, strongly suggests a progression toward sepsis. The characteristics of leukemoid reaction are listed in Box 16-3. Although viewed to be a clinically sensitive tool for predicting severe infection, these reactive changes have also been observed during uncomplicated pregnancy, massive trauma, inflammatory processes, drug reactions, and other toxic states.¹⁸ The function of circulating neutrophils may also be affected by infection. Both enhanced and impaired functions have been reported; however, it is generally accepted that mild infections enhance function, whereas severe infections impair function of neutrophil subsets.¹

BOX 16-3 Characteristics of Leukemoid Reaction

- Toxic granulation of neutrophil
- Vacuolization of neutrophil
- Döhle bodies in the cytoplasm of neutrophils
- "Shift to the left" increase in bands and metamyelocytes most often
- Leukocytosis usually greater than 30,000/mm³ ($>30 \times 10^9/L$); may be higher in young children; may be greater than 50,000/mm³ ($50 \times 10^9/L$) in patients receiving colony-stimulating factor therapy

Neutropenia Neutropenia is defined as an absolute decrease in the number of circulating neutrophils. The patient's age and race must both be considered in diagnosing neutropenia. Newborns and infants have a higher absolute neutrophil count; therefore, neutropenia is defined as an absolute count of less than $2,500/\text{mm}^3$ or $2.5 \times 10^9/\text{L}$. In addition, approximately 25% of individuals with African or Middle Eastern ancestry demonstrate benign ethnic neutropenia, with absolute neutrophil counts of 1.0 to $1.5 \times 10^9/\text{L}$.²⁰ True neutropenia can range from mild, with absolute counts from 1.0 to $1.5 \times 10^9/\text{L}$; to moderate, with counts from 0.5 to $1.0 \times 10^9/\text{L}$; to severe, with counts less than $0.5 \times 10^9/\text{L}$ (Table 16-3). Recurrent bacterial infections are the hallmark of persistent neutropenia, with the clinical severity reflected by the absolute neutrophil count as well as the frequency and duration of neutropenic episodes. Severe neutropenia is clinically significant, with risk of opportunistic, life-threatening infections when absolute neutrophil counts fall below $0.2 \times 10^9/\text{L}$, a state termed agranulocytosis.¹⁴

Infections in the neutropenic patient are most commonly caused by endogenous normal flora. Bacterial infections include *Staphylococcus aureus*, *Staphylococcus epidermidis*, streptococci, enterococci, *Pseudomonas aeruginosa*, and gram-negative enteric organisms; fungal infections are characterized by *Candida* or *Aspergillus* species; and viral infections are less common.^{13,15} Most infections in the neutropenic individual occur in the respiratory tract, cutaneous, and soft tissues. The major concern for these infections is the spread of the organism from the localized site into the bloodstream, resulting in a superinfection such as septicemia. Symptoms of infection can be atypical and nonpurulent due to decreased localized immune response.¹³

Neutropenic disorders are described as acquired or congenital. Persistent cases of neutropenia are generally attributed to an intrinsic problem of the hematopoietic system, whereas transient conditions are linked to factors extrinsic to the bone marrow. The absolute reduction in the circulating number of neutrophils may be attributed to increased peripheral destruction, decreased production (proliferation defect), impaired bone marrow release (maturation defect), or abnormal distribution. Identification of the cause is important for selecting appropriate therapy, monitoring prognosis, and counseling family members in congenital cases. The pathogenesis of neutropenia is outlined in Box 16-4.

Acquired Neutropenia Most acquired neutropenias occur as transient conditions caused by factors extrinsic to the bone

BOX 16-4 Pathogenesis of Neutropenia

- **Increased Destruction or Removal of Neutrophils**
 - Infections
 - Immune disorders
- **Maturation Defect**
 - Megaloblastic anemia
- **Proliferation Defect**
 - Aplastic anemia
 - Bone marrow replacement disorders
 - Idiosyncratic drug reactions
 - Myeloablative therapy
 - Bone marrow fibrosis
- **Abnormal Distribution**
 - Hypersplenism

marrow. In order of frequency, concomitant viral infection, the ingestion of certain medications, and alloantibody or autoantibody activity are all reported as causes.²¹ Disorders persisting longer than 3 months are considered chronic.²² (The drugs associated with causing neutropenia are listed in Table 16-4.) Neutropenia may also be acquired as a secondary condition to processes such as aplastic anemia, malignancy of the bone marrow, and dietary B₁₂ or folate deficiency (see Chapters 8 and 18-24). The causes of acquired neutropenia include:

- Infections
- Idiosyncratic drug reactions
- Autoimmune disorders
- Immunodeficiency disorders
- Hypersplenism
- Aplastic anemia
- Megaloblastic anemia
- Malignant disorders replacing the bone marrow

During times of stress or infection, the absolute neutrophil count for patients with acquired disorders can rise to levels consistent with mild neutropenia or into the normal range, revealing mobilization from existing bone marrow storage pools and offering differentiation from more severe, congenital disorders.¹⁴

TABLE 16-3 Classification of Neutropenia

Severity	Count
Mild	1,000–1,500/mm ³ (1.0 – $1.5 \times 10^9/\text{L}$)
Moderate	500–999/mm ³ (0.5 – $1.0 \times 10^9/\text{L}$)
Severe	< 500/mm ³ ($< 0.5 \times 10^9/\text{L}$)

TABLE 16-4 Drugs Associated With Causing Neutropenia

Drug Class	Drug Prototype
Antibiotics	Penicillin
	Chloramphenicol
Anti-inflammatory	Ibuprofen
Anticonvulsants	Phenytoin
Antithyroid	Propylthiouracil
Cardiovascular	Procainamide
Hypoglycemic	Chlorpropamide
Tranquilizer	Phenothiazine

Infection

The cause of neutropenia during infection is multifactorial; virus can directly suppress progenitor cell proliferation and differentiation by infecting hematologic precursors, or indirectly suppress proliferation through the induction of apoptosis promoting cytokines.²³ Viral infections are recognized as the most common cause of transient neutropenia, especially in children. The neutropenia appears during the first few days of illness when the peak level of virus is reached and lymphocytosis develops.

ADVANCED CONTENT

Specific virus may go unidentified in subclinical infections, while marked suppression is documented for infection by Epstein-Barr virus, influenza, cytomegalovirus, parvovirus B19, varicella, and respiratory syncytial virus.^{14,23} Other infections implicated include *Anaplasma*, *Brucella*, *Ehrlichia*, *Mycobacterium tuberculosis*, tularemia, typhoid, *Plasmodium* sp., or overwhelming sepsis with any organism.¹⁴

Immune-Mediated Neutropenia

Neutropenia may result from the activity of antineutrophil antibodies that mediate the removal of opsonized neutrophils from circulation. Immune-mediated neutropenia can be caused by either alloantibody or autoantibody, and patients present with absolute neutropenia that varies from $1.5 \times 10^9/L$ (mild) to less than $0.5 \times 10^9/L$ (severe).²⁴

Neonatal alloimmune neutropenia develops after maternal sensitization to fetal neutrophilic antigens. The antigens are shared by the fetus and the father, but they are absent in the mother. During pregnancy, antineutrophil antibody production is stimulated in the maternal immune system, and the IgG antibodies cross the placenta, destroying fetal neutrophils. The reduced absolute neutrophil count is typically present at birth and persists for approximately 6 months.²⁵ Alloantibodies may be directed against neutrophil specific antigens or HLA antigens that are shared by neutrophils and other nucleated cells.²⁵

ADVANCED CONTENT

There are currently 14 human neutrophil antigen alleles recognized across five antigenic systems (categorized as HNA-1 through HNA-5).²⁶ Diagnosis of neonatal alloimmune neutropenia is rare. Screening studies, although limited in number in size, have demonstrated granulocyte-specific antibodies present in 0.35% to 1.1% of random postnatal maternal samples with a corresponding incidence of developing neonatal alloimmune neutropenia below 0.1%.²⁵ Exact incidence cannot be known because cases can be asymptomatic, assays for detecting antibodies have shown limited sensitivity, and serological investigation by clinicians remains uncommon.^{25,27}

Autoantibodies directed against neutrophils have also been detected in the serum of neutropenic patients. When present,

these antibodies attach to the neutrophils of a patient and act as an opsonin for splenic removal. Autoimmune neutropenia can be classified as primary neutropenia of idiopathic origin, or as secondary to infection, immunodeficiency, autoimmunity, or malignancy; posthematopoietic stem cell transplant; or as a side effect of drug therapy.²⁷ Autoimmune neutropenia has been identified in both adults and children, although it is observed more frequently as the cause of chronic benign neutropenia of childhood, occurring most frequently in children under 4 years of age and self-resolving within 2 years of onset.²⁴ In adults, autoimmune neutropenia does not commonly resolve without intervention and is more commonly diagnosed as a secondary condition.^{24,26} Although no standard treatment has been established, antibiotics are used to treat specific bacterial infections and prednisone to limit the autoimmune response.^{14,27}

Congenital Neutropenia

Congenital neutropenias occur as chronic or intermittent disorders arising from defective or deficient production is intrinsic to the bone marrow microenvironment. Congenital defects are extremely rare and genetically heterogeneous, involving more than 24 genes affecting cells of the myeloid line or hematopoietic regulation.^{28,29} The disorders of congenital neutropenia include:

- Chronic benign neutropenia
- Severe congenital neutropenia (Kostman's)
- Myelokathexis
- Cyclic neutropenia
- Reticular dysgenesis
- Fanconi's anemia
- Dyskeratosis congenita
- Shwachman-Diamond syndrome

The consequences of these defects result in decreased or arrested cell production, or impaired release by the bone marrow. Clinical presentation varies from a mild, if any, increased risk of infection (as seen in chronic benign neutropenia) to the overwhelming, recurrent infections in severe congenital neutropenia that result in death within the first year of life. In severe congenital cases, clinical benefits have been seen with the administration of granulocyte-colony-stimulating factor (G-CSF) in terms of neutrophilic increment and infection prophylaxis.²⁹

Qualitative Disorders of Neutrophils

Disorders of Neutrophil Function Qualitative disorders of neutrophil function are characterized by bacterial infections that are caused by hereditary abnormalities in function. The occurrence of most qualitative conditions is extremely rare; many are familial and stem from a general metabolic defect. The exact pathophysiology of nearly all qualitative neutrophilic disorders is unknown. Neutropenia commonly accompanies these disorders. The qualitative disorders are classified according to the major defect expressed (i.e., cytoplasmic granules, disturbances of the respiratory burst, or chemotaxis); however, multiple abnormalities (including neutropenia) may be observed in some patients (Box 16-5).

Functional defects of neutrophils, which can be acquired or inherited, are classified by the general type of

BOX 16-5 Classes of Qualitative Neutrophil Disorders and Related Conditions

- **Cytoplasmic Granules**
 - Chediak-Higashi syndrome
 - Myeloperoxidase deficiency
 - Specific granule deficiency
- **Biochemical Disturbance of Respiratory Burst**
 - Chronic granulomatous disease
 - Glucose-6-phosphate dehydrogenase deficiency
 - Glutathione deficiency
- **Chemotaxis**
 - Lazy leukocyte syndrome
 - Monosomy-7

defect: (1) phagocytic/killing defects, (2) granule function and structure defects, (3) defects affecting chemotaxis and motility, and (4) adhesion defects.³⁰

The acquired defects in each category are listed in Box 16-6. The inherited disorders of neutrophil function are listed in Box 16-7. WBC counts are variable in neutrophil function defects depending on the nature of the disorders and whether the patient is actively infected. Automated analyzers do not flag qualitative WBC abnormalities. Examination of the peripheral blood smear is necessary for detection. Even then, examination of the peripheral blood smear is generally unremarkable in functional defects, with a few notable exceptions: the large granules that characterize Chediak-Higashi syndrome, the bilobed nuclei of Pelger-Huet anomaly, and absent secondary granules in neutrophil-specific granule-deficiency.

BOX 16-6 Acquired Disorders of Neutrophil Function**Phagocytic/Killing Defects**

1. Chronic infections
2. Autoimmune disorders
3. Diabetes
4. Cirrhosis
5. Splenectomy
6. GVHD
7. Malnutrition
8. Sickle cell anemia
9. Bone marrow transplant
10. Thermal injury

Granule Function and Structure Defects

1. Myeloid malignancies (CMPD, AML, myelodysplastic syndrome)
2. Pregnancy
3. Severe thermal injury
4. Trauma and surgery

Chemotaxis and Motility Defects

1. Autoimmune disorders
2. Numerous drugs and anti-inflammatory medications
3. Diabetes
4. Cirrhosis
5. Renal failure
6. Chronic infections
7. GVHD
8. Thermal injury
9. Malnutrition
10. PNH
11. Bone marrow transplant
12. Hematologic malignancies
13. Recombinant interleukin-2 therapy

Adhesion Defects

- Aging
- Drugs (i.e., aspirin, corticosteroids, epinephrine)
- Chronic infections
- Renal disorders
- Alcoholism
- Sickle cell anemia
- Paraproteinemia
- Diabetes

GVHD = graft-versus-host disease; CMPD = chronic myeloproliferative disorders; AML = acute myelocytic leukemia; PNH = paroxysmal nocturnal hemoglobinuria.

BOX 16-7 Inherited Disorders of Neutrophil Function**Phagocytic/Killing Defects**

- Chronic granulomatous disease
- Immunodeficiency disorders with decreased immunoglobulins
- Complement disorders
- Myeloperoxidase
- Specific granule deficiency

Granule Structure and Function Defects

- Chediak-Higashi syndrome
- Myeloperoxidase deficiency
- Specific granule deficiency

Chemotaxis and Motility Defects

- Lipid storage diseases (i.e., Gaucher's)
- Complement disorders
- Chediak-Higashi syndrome
- Specific granule deficiency
- Actin and microtubular defects
- Hyperimmunoglobulin E syndrome

Adhesion Defects

- Type 1, 2, and 3 leukocyte adhesion deficiencies

disorders. Special laboratory tests are necessary to evaluate neutrophil function. Molecular methods are used to diagnose disorders that arise from known genetic mutations. For practical purposes, the most clearly understood qualitative disorders are addressed here.

Chediak-Higashi Syndrome

Chediak-Higashi syndrome is a rare autosomal-recessive lysosomal storage disease characterized by neutrophils with abnormal function and morphology. The disorder is caused by mutations in the *LYST* gene that codes for the lysosomal trafficking regulator protein. Patients with Chediak-Higashi syndrome present with recurrent bacterial infections, oculocutaneous albinism, progressive neurological complications, and mild bleeding tendencies related to platelet granule defects³¹ (Figs. 16-7 and 16-8). Mutations in the *LYST* gene cause defective degranulation and result in the presence of giant lysosomal granules in cells such as granulocytes, monocytes, lymphocytes, melanocytes, tissue macrophages, and platelets.³²

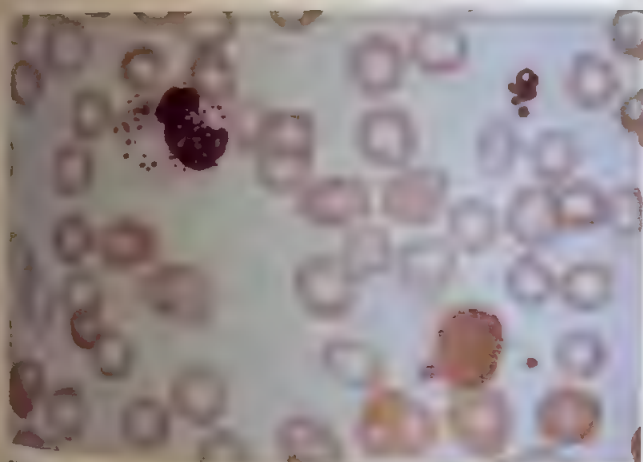


FIGURE 16-7 Peripheral blood from a patient with Chediak-Higashi syndrome. (Right) Lymphocyte. (Left) Neutrophil. (From Dutcher T. Hematology. In Listen, Look, and Learn, Bethesda, MD: Health and Education Resources, Inc., with permission.)

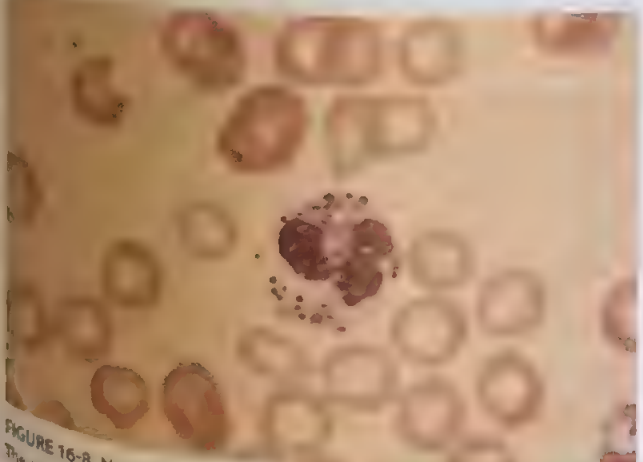


FIGURE 16-8 Neutrophil from a patient with Chediak-Higashi syndrome. The cytoplasm is filled with strikingly large primary (azurophilic) granules. (From Dutcher T. Hematology. In Listen, Look, and Learn, Bethesda, MD: Health and Education Resources, Inc., with permission.)

Many affected cells die within the bone marrow due to the defective cytoplasmic granulation. In addition to neutropenia, inefficient and prolonged bacterial killing has been identified as a key dysfunction in phagocytic activity. The release of lysosomal enzymes is impaired by the abnormal granule membrane fusion that results in the characteristic giant lysosomal granules. These abnormal granules develop during early myelopoiesis because of an initial aggregation of primary granules followed by fusion with the secondary granules³² (see Fig. 16-8). The giant lysosomal granules are more evident in cells of the bone marrow than in peripheral white cells. In circulation, the most striking granules may be seen in natural killer cells (NK subset of lymphocytes).³³ The granules stain from dark red to deep purple with Wright's stain and are strongly positive for peroxidase.

Many patients die as a result of infection during early childhood. Those who survive into early childhood enter an accelerated phase that progresses through pancytopenia, lymphoma-like cell infiltration, organomegaly, systemic infections, and eventually death.³¹ Management of Chediak-Higashi syndrome includes prophylactic antimicrobial therapy, and in the case of infection, aggressive intravenous treatment is required. The Epstein-Barr virus (EBV) is believed to trigger the accelerated phase of Chediak-Higashi syndrome, so immunization against EBV may be beneficial.³⁴ Hematopoietic stem cell transplant is the only curative treatment and is optimally performed before the onset of the accelerated phase or during remission.³⁴

Chronic Granulomatous Disease

Chronic granulomatous disease (CGD) is a familial, heterogeneous disorder of the neutrophil that may be chronic or intermittent. The mode of inheritance of most cases is X-linked recessive; however, autosomal recessive cases have been described.^{35,36} CGD is seen in 1 out of every 250,000 individuals, with patients generally diagnosed during the first two years of life. CGD is attributed to a failure in the activation of the respiratory burst that results in little or no superoxide production as the result of mutations in the genes encoding the phagocytic oxidase subunits (*phox*) of the enzyme, NADPH oxidase.³⁷ The interaction of these subunits results in the formation of superoxide during the respiratory burst.

ADVANCED CONTENT

Each *phox* subunit is identified as a glycoprotein (gp) or a protein (p). Two of the subunits, gp91-*phox* and p22-*phox*, are located in the plasma membrane and together form the enzyme cytochrome b. The other subunits, p47-*phox*, p67-*phox*, and p40-*phox*, reside in the cytosol. Stimulation of NADPH oxidase leads to the migration of the cytosol subunits to the plasma membrane. With the help of secondary mediators, the *phox* subunits assemble into the active oxidant, superoxide.³⁷ The classic X-linked form demonstrates a total absence of cytochrome b, whereas most autosomal-recessive cases present with deficiencies in one of the cytosol subunits.^{35,36} The molecular basis of WBC disorders is outlined in Table 16-5.

TABLE 16-5 Molecular Basis of WBC Disorders

Disorder	Inheritance	Affected Gene	Function
Chediak-Higashi syndrome	Autosomal recessive	LYST	Lysosomal trafficking regulator protein
Chronic Granulomatous Disease	X-linked	CYBB	gp91-phox NADPH oxidase subunit; integral membrane glycoprotein
Chronic Granulomatous Disease	Autosomal recessive	CYBA	p22-phox NADPH oxidase subunit; integral membrane protein
Chronic Granulomatous Disease	Autosomal recessive	NCF1	p47-phox NADPH oxidase subunit; cytosolic protein
Chronic Granulomatous Disease	Autosomal recessive	NCF2	p67-phox NADPH oxidase subunit; cytosolic protein
Chronic Granulomatous Disease	Autosomal recessive	NCF4	p40-phox NADPH oxidase subunit; cytosolic protein
Pelger-Huet	Autosomal dominant	LBR	Lamin B Receptor
May-Hegglin	Autosomal dominant	MHY9	nonmuscle myosin IIA heavy chain
Myeloperoxidase Deficiency	Autosomal recessive	MPO	myeloperoxidase
Leukocyte Adhesion Deficiency-I	Autosomal recessive	IGTB2	CD18 subunit of β_2 integrins
Leukocyte Adhesion Deficiency-II	Autosomal recessive	SLC35C1	GDP-fucose transporter 1
Leukocyte Adhesion Deficiency-III	Autosomal recessive	FERMT3	kindlin-3

Regardless of the subunit defect, superoxide cannot be produced, and, in turn, bacterial killing is ineffective. Ingestion of bacteria, degranulation, and phagolysosome formation are normal. Despite characteristic neutrophilia, patients with CGD are plagued by recurrent, life-threatening bacterial and fungal infections in nonsterile sites, which spread hematogenously to other organs and deep tissue. The hallmark of CGD is the formation of granulomas during chronic inflammatory reactions that keep the organisms localized.³⁸ Most common types of infections include pneumonia, lymphadenitis, and abscesses. The most common agents of infection in the United States and Europe include *Staphylococcus aureus*, *Serratia marcescens*, *Salmonella* sp., *Klebsiella* sp., *Nocardia* sp., and *Aspergillus* sp.; *Mycobacterium tuberculosis* is an additional cause of mortality for patients in parts of the world where infection is endemic within the general population.³⁸⁻⁴⁰

Diagnosis is established by demonstrating a bactericidal defect resulting from the absence of the oxidative burst using the nitroblue tetrazolium (NBT) slide test, cytochrome *c* reduction measured by spectrophotometry, or by measuring respiratory burst activity with flow cytometry.³⁶ The NBT slide test is used to indirectly detect the production of superoxide through the reduction of the NBT dye and the accumulation of a blue-black precipitate, recorded as a positive reaction microscopically. The NBT test has generally been replaced by flow cytometry methods that use dyes sensitive to the generation of reactive oxygen species, such as dihydrorhodamine 123.³⁶ The X-linked-recessive inheritance may be confirmed by studying the family history and performing molecular gene analysis. Indicators of CGD include the presence of disease in male members of the maternal family and intermediate to low neutrophil activity in the mothers and sisters of affected boys. In most cases, the female relatives are clinically well, but occasionally they may present with an increased

susceptibility to infections or a syndrome resembling systemic lupus erythematosus.^{35,36}

Progress has been made in modalities of treatment, and the prognosis of CGD is improving. Aggressive prophylactic antibiotic therapy should be initiated as soon as a diagnosis is made. Gamma (γ) interferon has been effective in limiting the frequency of infections, and granulocyte transfusions are useful in patients who respond poorly.³⁹ Hematopoietic stem cell transplant is the main curative treatment for patients with CGD and has been proven successful in over 90% of patients; however, the rate of success is dependent on the HLA-match and supportive therapy.⁴

Myeloperoxidase Deficiency

Myeloperoxidase (MPO) deficiency is the most common inherited disorder of phagocytes. As an important component of neutrophilic granules, myeloperoxidase deficiency inhibits the formation of hypochlorous acid during phagocytic killing, impeding ingestion by phagocytes.⁴² Despite this deficiency, patients are rarely symptomatic, and diagnosis may be secondary to the laboratory investigation of a different disorder. MPO deficiency notably presents as false pseudoneutropenia when using automated analyzers that rely upon myeloperoxidase presence for the detection of neutrophils.⁴² Deficiency is inherited as an autosomal recessive defect in the MPO gene.⁴³ Myeloperoxidase activity can be evaluated microscopically using histochemical staining or quantitated using spectrophotometry methods (Chapters 31 and 34-35). MPO deficiency must be differentiated from the diagnosis of CGD using the DHR flow cytometry assay, which will be abnormal in both conditions; NBT slide assays are unaffected in MPO-deficient patients.⁴¹

Leukocyte Adhesion Deficiency

Leukocyte adhesion deficiency (LAD) is an autosomal recessive disorder, most commonly defined by mutations in *CD18*, a common chain of the β_2 integrin family of glycoproteins.

ADVANCED CONTENT

Currently, there are three categories of LAD, of which LAD-I is the most common disorder. LAD-I is caused by a mutation in *ITGB2*, the gene coding for CD18. Defective CD18 expression results in the lost expression of three distinct β_2 integrins: CD11a/CD18, CD11b/CD18, and CD11c/CD18.³⁸ This loss results in neutrophilia with impeded migration of neutrophils to sites of inflammation or infection. Patients are subject to recurrent bacterial and fungal infections.^{38,45} Diagnosis using flow cytometry to demonstrate the loss of expression is straightforward and, combined with the identification of genetic defect, can distinguish LAD subtypes.⁴⁵

Disorders of Neutrophil Morphology Granulocytes with abnormal morphology can be acquired or inherited. The acquired disorders of neutrophil morphology are listed in Box 16-8. Although rare, several hereditary abnormalities in neutrophil morphology may be observed in patients without an association with infection, neutrophilic dysfunction, or altered neutrophil number. Such abnormalities in neutrophilic morphology are collectively referred to as “white blood cell anomalies.” The inherited disorders of neutrophil morphology are listed and their characteristics summarized in Box 16-9.

Nuclear Anomalies

Hypersegmentation and hyposegmentation of the nucleus are WBC anomalies that reflect the number of segmented lobes demonstrated in the mature neutrophil. Hypersegmentation describes larger-than-normal neutrophils with six or more nuclear lobes present. A similar hypersegmentation of eosinophils has been reported, with five or more nuclear lobes present. Hypersegmentation in the granulocytes may be an indicator of an acquired anemia, such as megaloblastic anemia, or of a benign autosomal-dominant condition known as hereditary hypersegmentation of neutrophils.⁴⁶

Hyposegmentation of the nucleus is characteristic of Pelger-Huët anomaly, a condition that affects all leukocytes

BOX 16-9 Inherited Disorders of Neutrophil Morphology

- **Pelger-Huët Anomaly**
 - Autosomal dominant
 - Bilobed or nonsegmented nuclei
 - Normal cytoplasm
- **May-Hegglin Anomaly**
 - Autosomal dominant
 - Large blue cytoplasmic inclusions found in neutrophils, eosinophils, basophils, and monocytes
- **Chediak-Higashi Syndrome**
 - Autosomal recessive
 - Giant cytoplasmic granules in granulocytes and lymphocytes
- **Alder-Reilly Anomaly**
 - Autosomal recessive
 - Cytoplasm of neutrophils contain dark azurophilic granules
 - Eosinophils and basophils also affected
 - Lymphocytes may also be abnormally granulated and vacuolated
- **Specific Granule Deficiency**
 - Autosomal recessive
 - Absence of secondary granules, neutrophils appear pale and hyposegmented
 - Abnormal granules in platelets and eosinophils

but is identifiable in neutrophils in which the nucleus is found to be bilobed or to have no lobulation whatsoever, with exceptionally coarse and condensed nuclear chromatin. Despite the distinguishable morphology, the neutrophils appear to function normally. In the heterozygous state, predominantly bilobed neutrophilic forms are present that are described as having a “dumbbell” or “pince-nez” appearance with two symmetric lobes being joined by a filament. In the homozygous state, no segmentation is evident, and the nucleus takes on a round or oval appearance, occasionally referred to as the “Stodmeister” form⁴⁷ (Fig. 16-9). “True” Pelger-Huët anomaly is inherited in an autosomal-dominant pattern; heterozygous expression is observed in 1 out of 6,000 individuals, yet homozygous expression is exceedingly rare.⁴⁷

BOX 16-8 Acquired Disorders of Neutrophil Morphology

- **Hypersegmentation**
 - Megaloblastic anemia
 - Myeloid malignancies (i.e., AML, myelodysplastic syndromes)
- **Hypogranularity**
 - Myeloid malignancies (i.e., AML, myelodysplastic syndromes)
- **Pseudo-Pelger-Huët**
 - Myeloid malignancies (i.e., AML, myelodysplastic syndromes)
 - Drugs (sulfonamides, colchicines, etc.)
- **Cytoplasmic Inclusions**
 - Cryoglobulinemia
- **Giant Neutrophils**
 - AIDS infection
 - Granulocyte Colony Stimulating Factor (G-CSF) therapy

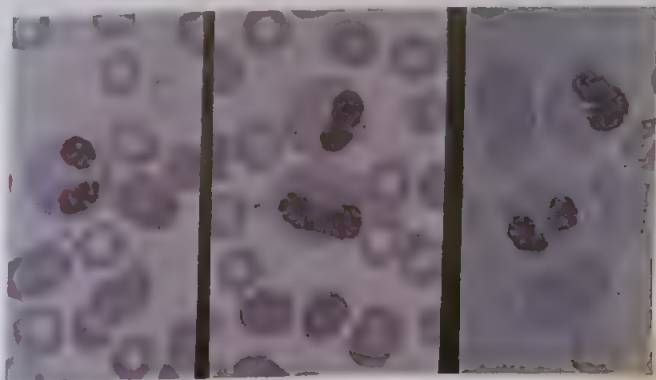


FIGURE 16-9 Pelger-Huët anomaly (peripheral blood). (From Hyun BH, et al. Practical Hematology. A Laboratory Guide With Accompanying Filmstrip. Philadelphia: WB Saunders; 1975, with permission.)

ADVANCED CONTENT

The inherited genetic abnormality affects the *LBR* gene, encoding the lamin B receptor. This protein resides in the membrane, and the N-terminus interacts with chromatin and the nuclear lamina of the cell.⁴⁸

Acquired or pseudo Pelger-Huët neutrophils have similar morphology but are induced by drug ingestion or occur secondary to conditions such as leukemia. Differentiating between acquired and true Pelger-Huët can be difficult. One way is to look at the percentage of cells observed; true Pelger-Huët neutrophils will be observed in >50% of the total neutrophil count and acquired Pelger-Huët in <30%.⁴⁹ Care must be taken to distinguish Pelger-Huët cells from a “shift to the left,” in which an increase in metamyelocytes and bands is observed during severe infection. Generally, the nuclear chromatin is more condensed and coarse in Pelger-Huët neutrophils than in bands and metamyelocytes.

Cytoplasmic Anomalies

Morphological changes may also be noted in the neutrophilic cytoplasm. The presence of prominent, dark-staining, coarse cytoplasmic granules in neutrophils, eosinophils, basophils, monocytes, or occasionally lymphocytes is known as **Alder’s anomaly or Alder-Reilly inclusions** (Fig. 16-10). In some patients, only one cell line may be affected. These cytoplasmic inclusions are composed of precipitated mucopolysaccharide and are seen in association with inherited disorders of mucopolysaccharidosis, such as Hunter’s and Hurler’s syndromes, where a genetic defect affects the lysosomal degradation of glycosaminoglycans. Inheritance of such disorders is typically autosomal recessive⁵⁰ (see Chapter 24). These prominent granules are similar to those in toxic granulation, with the exceptions that they are larger, stain positive with metachromatic stains, and are a permanent morphological characteristic of the neutrophils.

As discussed previously, toxic granulation is an acquired transient change in morphology caused by infectious or toxic agents, often accompanied by the presence of Döhle bodies.

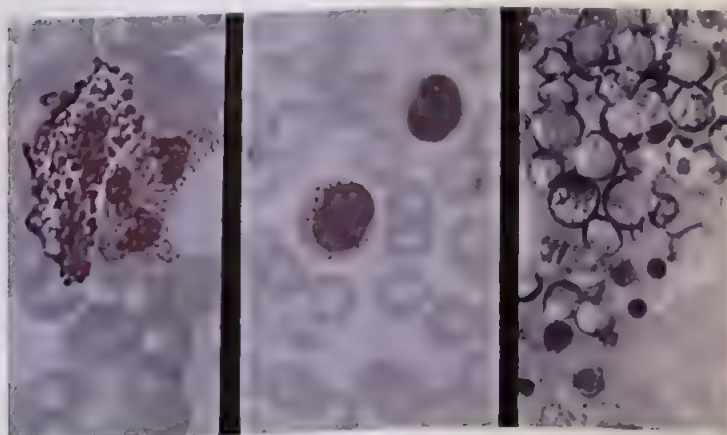


FIGURE 16-10 Alder-Reilly anomaly. (Left and middle) Note azurophilic granulation in cells from peripheral blood. (Right) Bone marrow. (From Hyun BH, et al. *Practical Hematology. A Laboratory Guide With Accompanying Filmstrips*. Philadelphia: WB Saunders; 1975, with permission.)

Granulocytes and monocytes in **May-Hegglin anomaly** demonstrate larger, blue-staining cytoplasmic inclusions that resemble Döhle bodies⁵¹ (Fig. 16-11).

ADVANCED CONTENT

May-Hegglin is a hereditary macrothrombocytopenia characterized by mutations in the *MYH9* gene, causing defects in nonmuscle myosin IIA heavy chain, affecting megakaryocytic migration and platelet release.⁵² *MYH9* disorders are typically inherited in an autosomal dominant fashion; approximately one-third occur due to sporadic mutations.⁵² The inclusions present in May-Hegglin are composed of defective nonmuscle myosin IIA protein, *MYH9* mRNA, and ribosomes.⁵³

This anomaly is also associated with thrombocytopenia and giant platelets (macrothrombocytopenia) and show variable neutropenia and bleeding tendency. Many patients may be clinically asymptomatic (see Chapter 26).

Eosinophils

The normal percent of eosinophils found upon examination on the peripheral blood smear is approximately 2% to 4%. Eosinophils function to release their secondary granules, rich in major basic protein, to destroy parasites and function in immediate hypersensitivity reactions upon migration to the tissues. Eosinophilia occurs when the absolute eosinophil count is greater than 500 cells/mm³ or 0.5×10^9 cells/L. There are no age-related variations in the absolute counts. Eosinophilia can be arbitrarily classified using the absolute count; the absolute counts for mild, moderate, and severe eosinophilia are listed in Table 16-6.⁵⁴

Causes of eosinophilia can be categorized as neoplastic, reactive, and idiopathic.⁵⁴ The most common cause of eosinophilia worldwide is parasitic infections. To produce



FIGURE 16-11 May-Hegglin anomaly. Note the Döhle body present in each neutrophil (arrows). Not shown in the slide but associated with May-Hegglin anomaly is the presence of giant platelets. (From Dutton J. *Hematology. In Listen, Look, and Learn*. Bethesda, MD: Health and Education Resources, Inc., with permission.)

TABLE 16-8 Classification of Eosinophilia

Severity	Count
Mild	600–1,500/mm ³ (0.6–1.5 × 10 ⁹ /L)
Moderate	1,500–4,900/mm ³ (1.5–4.9 × 10 ⁹ /L)
Severe	>5,000/mm ³ (>5.0 × 10 ⁹ /L)

eosinophilia, the parasites must invade the tissues. Helminthic infection is the most common parasite causing eosinophilia. In developed countries, allergic disorders are the most common causes of reactive eosinophilia. The causes of secondary reactive eosinophilia are listed in Box 16-10. Common characteristics of acquired secondary reactive eosinophilia include:

- Mild to moderate eosinophilia
- Represents a compensatory bone marrow response to an increased tissue demand for eosinophils
- Eosinophilia disappears with the resolution of the causative disease

Causes of mild to moderate eosinophilia detected by a routine complete blood count often occur without any known cause.⁵⁴

BOX 16-10 Causes of Secondary (Nonmalignant) Reactive Eosinophilia (Absolute Count > 600/mm³ or 0.6 × 10⁹/L)

- **Allergic Disorders**
 - Asthma
 - Urticaria
 - Allergic rhinitis
- **Parasitic Infections**
 - Helminthic (must invade tissue to produce eosinophilia)
- **Skin Disorders**
 - Bullous pemphigoid
 - Pemphigus
 - Atopic dermatitis
 - Eczema
- **Pulmonary Disorders**
 - Loeffler syndrome
 - Bronchiectasis
 - Pneumonia
 - Cystic fibrosis
 - Churg–Strauss syndrome
- **Other Inflammatory Disorders**
 - Celiac disease
 - Vasculitides
 - Inflammatory bowel disease
 - Collagen vascular disorder
 - Sarcoidosis

ADVANCED CONTENT

In malignant hematopoietic disorders such as chronic myeloproliferative disease and acute leukemias, eosinophilia may be seen because they are part of the clone and often demonstrate morphological abnormalities. Clonal hypereosinophilia or genetic rearrangements of *PDGFRA*, *PDGFRB*, *FGFR1*, or *PCM-JAK2* are neoplastic and classified as myeloproliferative disorders by the World Health Organization^{54–56} (see Chapters 17–19).

Basophils

The normal percentage of basophils present upon examination of the peripheral blood smear is 1% to 2%. Basophilia occurs when the absolute count is greater than 100 cells/mm³ or 0.1 × 10⁹ cells/L.⁵⁷ Isolated basophilia is uncommon. Reactive conditions can cause moderately increased basophil counts, and uncommonly occur secondary to allergy, hypersensitivity, or inflammation due to the major component of basophilic granules being histamine. However, basophilia is an uncommon finding.¹⁸ The secondary reactive causes of basophilia are listed in Box 16-11.

More commonly, basophilia is indicative of a malignant process and characterized by absolute counts that are much higher due to the extreme elevation in WBC count characteristic of myeloproliferative neoplasms.⁵⁷ Chronic myelogenous leukemia (CML) is the most common cause of basophilia¹⁹ (see Chapter 18).

Monocytes

The normal percentage of monocytes present on examination of the peripheral smear is 2% to 9%, with an absolute count that may reach 1,500 monocytes/mm³ at birth, and gradually decreases to a range between 100 to 900 monocytes/mm³ or

BOX 16-11 Causes of Secondary Reactive Basophilia (Absolute Count > 200/mm³ or 0.2 × 10⁹/L)

- **Infections**
 - Chicken pox
 - Smallpox
 - Influenza
- **Inflammatory Disorders**
 - Rheumatoid arthritis
 - Ulcerative colitis
 - Collagen vascular disease
- **Allergic Reactions**
 - Urticaria
 - Allergies to food and drugs
 - Erythroderma
- **Chronic Renal Disease**
- **Endocrine Disorders**
 - Diabetes
 - Hypothyroidism
- **Exposures to Radiation**

0.1 to $0.9 \times 10^9/L$.⁵⁸ Beyond infancy, however, there are no age-related variations. Monocytes, on migration to the tissues, function in phagocytic and antimicrobial activities, tissue repair, and various other functions in cellular and humoral immunity. Monocytes contain numerous very fine granules in their cytoplasm that play a major role in destruction of microbes. The enzymes found in the granules of monocytes are lipzyme, collagenase, acid phosphatase, and elastase. Functions of the many proteins secreted by monocytes include:

- Regulation of hematopoiesis
- Stimulation of inflammatory reactions
- Removal of infectious organisms by phagocytosis
- Removal of senescent blood cells
- Modulation of the immune function
- Stimulation of host defense against tumor cells

Monocytosis occurs when the absolute monocyte count exceeds 1,000 monocytes/mm³ or $1.0 \times 10^9/L$.⁵⁸ Reactive monocytosis is generally associated with a chronic infection, autoimmune disease, splenectomy, and a range of malignancy.^{18,19} The causes of secondary reactive monocytosis are listed in Box 16-12. Absolute monocytosis can also be found in malignant hematopoietic disorders such as chronic myelomonocytic leukemia (CMML), chronic myelogenous leukemia (CML), acute monocytic leukemia, and acute myelomonocytic leukemia (AMML) (see Chapters 17 and 18).

Distinguishing reactive and neoplastic causes of monocytosis can be challenging, as persistent increases in cell number and morphological criteria remain as hallmarks of diagnosis under the most recent World Health Organization (WHO) criteria.^{56,59} Reactive monocytosis can present with additional abnormalities characteristic of the primary diagnosis. For example, monocytosis secondary to chronic infection may be accompanied by neutrophilia, leukocytosis, mild to moderate anemia, and variable platelet count. In contrast, patients

BOX 16-12 Causes of Secondary Reactive Absolute Monocytosis (Adults: Absolute Counts $>1.0 \times 10^9/L$; Newborns: $>1.2 \times 10^9/L$)

Chronic Infection

- Fungal
- Bacterial
- Protozoal
- Rickettsial

Inflammatory Conditions

- Sarcoidosis
- Collagen vascular disorders
- Inflammatory bowel diseases
- Sprue

Other

- Immune thrombocytopenic purpura (ITP)
- Hemolytic anemia
- Postsplenectomy

Chronic Neutropenia

- Acquired neutropenia
- Inherited cyclic neutropenia

Malignant Hematological Disorders

- Hodgkin lymphoma
- Non-Hodgkin lymphoma
- Multiple myeloma

with monocytosis, as a result of a malignant clone, present with more severe anemia and variable platelet and leukocyte counts.⁵⁸ The morphology of monocytes in reactive monocytosis shows relatively mature monocytes with clumped nuclear chromatin, which is present in a folded, indented, or unusually shaped nuclei. The cytoplasm is usually spread out and frequently vacuolated. Numerous dustlike fine granules, which are normally hard to see, may be very prominent in these reactive monocytes. In monocytes associated with a hematopoietic malignancy, more immature forms with fine nuclear chromatin and nucleoli are observed, usually without the reactive morphological features previously described. Demonstration of clonality or genetic mutation also distinguishes malignant and reactive monocytosis.⁵⁶

Lymphocytes

Lymphocytosis refers to an increase in lymphocytes in the peripheral blood. In adults, lymphocytes represent 20% to 40% of the relative differential count. Relative lymphocytosis refers to an increase in the percentage of lymphocytes when performing the WBC differential. Absolute lymphocytosis represents the total number of lymphocytes present and is calculated as a relative percentage of the overall WBC count. It is important to use accurate reference ranges by age when evaluating the absolute lymphocyte count, as the normal relative and absolute values shift to a predominance of lymphocytes after birth, remain elevated during childhood, and return to a predominance of neutrophils during adulthood.¹⁸ In infants and young children, absolute lymphocyte counts greater than $11.0 \times 10^9/L$ define lymphocytosis, while the threshold for absolute lymphocytosis exceeds $4.0 \times 10^9/L$ in adult patients.⁶⁰

Absolute Lymphocytosis: Reactive Versus Malignant Causes

The term **reactive lymphocytes** is used to describe transformed or benign lymphocytes. The term **atypical** should not be used interchangeably with reactive because, in pathology, atypical may imply malignant-appearing cells.⁶¹ Other terms that have been used to describe the spectrum of reactive lymphocytes include immunocytes, transformed lymphocytes, immunoblasts, plasmacytoid lymphocytes, Turk cells, and Downey cells. Reactive lymphocytes occur in normal patients but usually account for less than 10% of the total lymphocytes. Generally accepted review criteria maintains that slides with instrumentation flags for reactive lymphocytes should receive a manual review, and patients presenting with counts in excess of $5.0 \times 10^9/L$ are recommended for review by a pathologist.⁶² Control slides with reactive lymphocytes should be reviewed on a regular basis with staff to ensure uniformity in the reporting of reactive lymphocytes.

Lymphocyte Morphology

Most lymphocytes are small; however, intermediate and some large lymphocytes may be observed. Normal lymphocytes have a high nuclear-to-cytoplasmic ratio, with the nucleus being oval or round and the chromatin clumped and staining dark purple. The cytoplasm is blue, with varying intensity from light to dark in different cells. Although most lymphocytes

do not have granules, large lymphocytes may have a few well-defined purplish-red granules that can be easily counted at the periphery of the cytoplasm. Large granular lymphocytes (LGLs) are characterized by a few cytoplasmic granules (Fig. 16-12). These granules are azurophilic, appear red or purple, and are larger than the fine, pink, secondary granules present in neutrophils. LGLs may be of T-cell or true natural-killer cell origin. In contrast, the cytoplasm of monocytes contains many small granules and has a "ground-glass" cloudy appearance.

Reactive lymphocytes and normal lymphocytes vary in size, shape, and immunophenotypic markers (polyclonal) (Table 16-7). The cells do *not* originate from one precursor cell or clone. In contrast, lymphocytes in malignant disorders are similar with regard to size and shape (monomorphic) and immunophenotype, because they originate from the same malignant clone (monoclonal).¹⁸ Malignant lymphocytes may vary in size depending on the particular malignancy. However, for any one malignancy, the size and appearance tend to be constant.

Reactive lymphocytes range in size from 9 to 30 μm . The size variation that may be seen with reactive lymphocytes is illustrated in Figure 16-13. Resting small lymphocytes tend to be much smaller than reactive lymphocytes, ranging in size from 8 to 12 μm , and are similar in size to the top right lymphocyte in Figure 16-13 (right arrow). The ratio of the nuclear area to the visible cytoplasmic rim (i.e., the N:C ratio) varies with reactive lymphocytes. One of the most distinguishing features of reactive lymphocytes is the abundant cytoplasm, compared with smaller resting lymphocytes.⁶³ Resting small lymphocytes have relatively little cytoplasm, whereas NK (natural-killer) lymphocytes may have moderate amounts of cytoplasm, resulting in the terminology of "large granular lymphocytes" for these cells. In Figure 16-13, three lymphocytes are present. In the center of the field is a reactive lymphocyte, specifically a large granular lymphocyte with abundant cytoplasm (large arrow). The cytoplasm is usually pale blue, with occasional azurophilic granules. A significant morphological feature of reactive lymphocytes is the uneven staining of the cytoplasm. In reactive lymphocytes, peripheral portions of the cytoplasm often stain darker blue than areas

TABLE 16-7 Lymphocyte Morphologies

Characteristics	Reactive Lymphocyte	Resting Small Lymphocyte*
Size	Large (9–30 μm)	Small (8–12 μm)
N:C Ratio†	Low to moderate	High to moderate
Cytoplasm amount	Abundant	Scant
Cytoplasm Color	Colorless to dark blue	Colorless to light blue
Nucleus shape	Round to irregular	Round
Chromatin	Coarse to moderately fine‡	Coarse
Nucleoli	Absent to distinct	Absent
Typing	Polyclonal	Polyclonal

*Normal peripheral blood may contain a few medium-sized natural killer cells that are larger than resting small lymphocytes but smaller than large reactive lymphocytes.

†The N:C ratio is the ratio of the nuclear area to the visible cytoplasmic rim.

‡The chromatin is not as fine as that seen in blast cells.

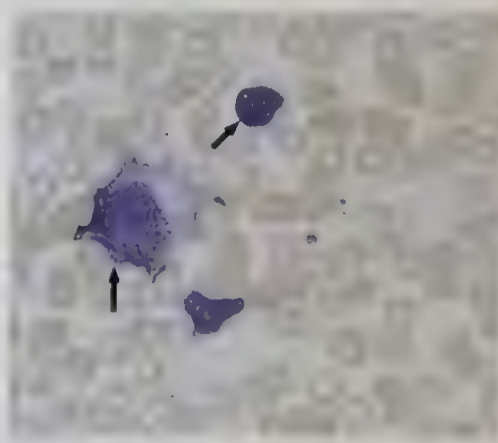


FIGURE 16-13 Three lymphocytes are present, one with abundant cytoplasm (low N:C ratio) and two with moderate amounts of cytoplasm, from a patient with infectious mononucleosis. Note the variation in the chromatin coarseness. The larger or reactive-appearing lymphocyte has prominent cytoplasm indentations (center arrow).

of the cytoplasm closer to the nucleus. Also, the cytoplasmic border is usually round with an occasional indentation.

The nucleus in reactive lymphocytes may be round, indented, or lobulated (Fig. 16-14). In reactive conditions, the appearance of the nuclear chromatin in the lymphocyte population is variable and not monotonous. Generally, the nuclear chromatin is coarse or clumped, and prominent clumping or coarseness of the chromatin similar to plasma cells may occur. A small "plasmacytoid" lymphocyte with prominent chromatin clumping, perinuclear halo, and an eccentric nucleus similar in appearance to a plasma cell is illustrated in Figure 16-15. The nucleus in resting small lymphocytes is round and less variable. The chromatin may vary in coarseness but not to the extent seen with reactive lymphocytes.

Nucleoli may be present in reactive lymphocytes. Such reactive lymphocytes may be differentiated from lymphoid

FIGURE 16-12 A large, mature-appearing lymphocyte with azurophilic granules.

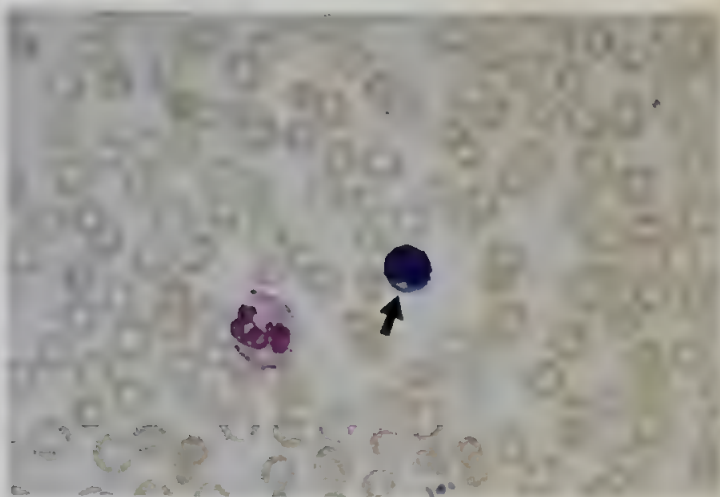


FIGURE 16-14 Lymphocyte with nuclear indentation.

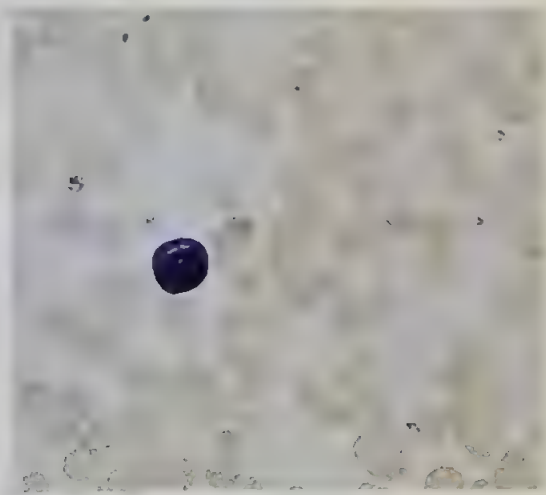


FIGURE 16-15 A small plasmacytoid lymphocyte with coarse chromatin and an eccentric nucleus.

or myeloid blasts by having more abundant cytoplasm (i.e., a lower N:C ratio) and a clumped chromatin pattern. A reactive lymphocyte with a prominent nucleolus and deeply basophilic cytoplasm, referred to as an “immunoblast,” is illustrated in Figure 16-16. **Immunoblast** is a descriptive morphological term and has no bearing on the lymphocyte type.

Nucleoli are not apparent in resting small lymphocytes. Reactive lymphocytes are compared with small resting lymphocytes (see Table 16-7).

Morphological criteria alone usually enable one to distinguish reactive lymphocytosis from malignant disorders. When there is ambiguity, clinical history, cell typing, and serological findings may help in distinguishing these entities.^{17,18} If necessary, a bone marrow or lymph node biopsy may lead to the correct diagnosis. The most common causes of a reactive lymphocytosis are shown in Box 16-13.

Causes of Reactive Lymphocytosis

Infectious Mononucleosis

History

In 1907, Turk described clinical symptoms in several patients who probably suffered from infectious mononucleosis (IM) caused by EBV infection.⁶⁴ Reactive lymphocyte morphology

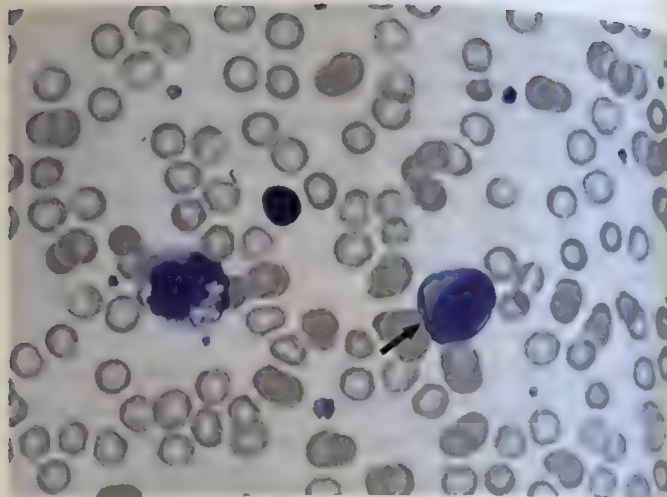


FIGURE 16-16 A large reactive lymphocyte with prominent nucleolus (immunoblast).

BOX 16-13 Causes of Reactive Lymphocytosis

- **Viral**
 - Adenovirus
 - Chickenpox
 - Cytomegalovirus
 - EBV (infectious mononucleosis)
 - Hepatitis
 - Herpes simplex
 - Herpes zoster
 - Human immunodeficiency virus (HIV)
 - Influenza
 - Paramyxovirus (mumps)
 - Rubella (measles)
- **Bacterial**
 - Brucellosis
 - Paratyphoid fever
 - Pertussis (whooping cough)
 - Tuberculosis
 - Typhoid fever
- **Drug Reactions**
 - During recovery from acute infections (especially in children)
- **Miscellaneous**
 - Acute infectious lymphocytosis
 - Allergic reactions
 - Autoimmune diseases
 - Hyperthyroidism
 - Malnutrition
 - Rickets
 - Syphilis
 - Toxoplasmosis

and clinical symptoms were correlated in 1920 by Sprunt, who named the syndrome “infectious mononucleosis.” Downey, in 1923, described in further detail the unusual lymphocyte morphology associated with IM.⁶⁶ Although morphology is still important, Downey’s descriptive terminology of morphology is seldom used today. *Infectious mononucleosis* is a historical term. We now know that the large mononuclear cells originally described in IM are lymphocytes and not monocytes.

Later, in 1932, Paul noticed that serum from patients with IM contained antibodies against sheep erythrocytes.⁶⁷ This discovery was the basis for the Monospot test (Ortho Pharmaceuticals). This contemporary test is the most commonly used and the simplest method available for measuring IgM heterophile antibodies. Heterophile antibodies are antibodies that also react with cells of other species. In particular, the IgM antibodies react with sheep and horse erythrocytes. The antibodies are not absorbed by guinea pig kidney in the Monospot test and, thus, if present, agglutinate horse erythrocytes.

The DNA virus responsible for IM was first observed in lymphoblasts cultured from patients with Burkitt's lymphoma⁶⁸ (see Chapter 21). This virus is now known as the Epstein-Barr virus (EBV). In addition to Burkitt's lymphoma, EBV has been associated with other malignancies, B-cell lymphoproliferative syndromes, and persistent clonal populations.⁶⁹ The malignancies appear to occur after the virus has been dormant in the host for years.

Regarding acute viral illness, EBV was first linked as the causative agent for IM by Henle in 1967.⁷⁰ He was able to establish a lymphocyte cell line from the blood of a laboratory worker who was infected with EBV and managed to show by serological studies that EBV was responsible for IM. Additional evidence supporting the role of EBV in IM has been provided by the detection of EBV nucleic acid sequences using polymerase chain reaction on lymphoid tissue from patients with IM.⁷¹ Unlike EBV-associated malignant conditions, IM results from primary infection.

ADVANCED CONTENT

EBV belongs to the herpes virus family. Humans can be infected by one of two strains of EBV, type A or B, which are widespread in North America and may coinfect the same individual.⁷² EBV primarily infects B lymphocytes and epithelial cells of the pharynx and cervical lymph nodes, which all share the CD21 receptor.⁷¹ Less than half of the patients who experience primary infection with EBV will develop IM.⁷³ The majority of atypical lymphocytes associated with IM have been identified as CD8⁺ cytotoxic lymphocytes, although T helper cells and NK cells are also present.⁷⁴

Clinical Findings

Infectious mononucleosis is more commonly present in young individuals, with the peak incidence between 17 and 25 years of age. The propensity of IM to develop during adolescence is thought to be related to the immune system's reaction to the virus.^{73,74} The virus enters the body orally through the lymphoid tissue in the pharynx and, in turn, infects B lymphocytes. The onset is generally abrupt, the most consistent initial symptoms being sore throat, lymphadenopathy, fever, dysphagia, general malaise, and excessive fatigue that may persist for 2 to 3 weeks. The severity of the sore throat pain leads to difficulty in swallowing and anorexia. Other commonly observed symptoms include nausea, headache, myalgia, sweats, and chills. Infrequently, individuals present with mild autoimmune hemolytic anemia because of cold-reacting

antibodies (agglutinins) specific for the "i" red blood cell antigen (see Chapter 13) and immune thrombocytopenia caused by increased splenic activity.

Multiple organ involvement with related symptoms may be present in IM. Enlargement of the anterior cervical lymph nodes is another physical finding during the first week. Following a week or two, the swelling usually subsides. The nodes are firm but not tender or warm. At least one-half of patients demonstrate a palpable spleen during the course of infection, and, rarely, splenic rupture may occur. Hypersplenism may contribute to mild anemia, immune thrombocytopenia, or both. Hepatomegaly may be detected in up to 25% of patients with IM. Liver enzymes and bilirubin levels may be elevated because of liver involvement.

Infectious mononucleosis is uncommon in older adults and, therefore, the differential diagnosis of lymphocytosis is influenced by the age of the patient. For example, in a 50-year-old patient with increasing numbers of benign-appearing lymphocytes, the diagnosis of chronic lymphocytic leukemia (CLL) is more likely than IM; however, in a child or young adult, CLL is extremely rare. It should be noted that many children contract IM in early childhood, which is misdiagnosed as a bad cold or flu. In most cases, the development of antibodies results in long-term immunity.

Laboratory Results

The differential diagnosis for IM includes a variety of entities, which can be narrowed considerably by evaluation of a peripheral blood smear and serological findings. The skilled morphologist should be able to readily differentiate the reactive lymphocytes seen in IM from malignant lymphoid cells seen in leukemia or lymphoma. Cytomegalovirus, rubella, hepatitis, and other viral illnesses may have reactive lymphocytes and may require additional serological findings to distinguish them from EBV infection.⁶³ Acute streptococcal pharyngitis, diphtheria, and other bacterial infections are identifiable by culture, and the lymphocytes usually do not have the reactive morphology seen in viral infections. Classical reactive lymphocytes and definitive serological findings may not always be present; therefore, it may be difficult in some instances to distinguish between IM and other processes.

ADVANCED CONTENT

In the absence of positive heterophile antibody (monospot test), testing for EBV specific antibodies by immunoassay is valuable; the most useful antibodies are VCA IgM, VCA IgG, and EBNA-1 IgG.⁷¹ In rare cases, additional studies, such as lymph node biopsy, may be indicated.⁷⁵

Treatment, Clinical Course, and Prognosis

Treatment of IM is mostly symptomatic, with some patients requiring bedrest. Little progress has been made in decreasing the duration of illness, with antiviral therapies or interferon to treat complications showing few benefits.⁷¹ In severe cases marked by airway obstruction or corresponding autoimmune phenomena (thrombocytopenia or anemia), corticosteroids

have been used as treatment, although their success remains controversial.⁷¹ Antibiotics are not useful unless there are complications or coinfection. In acute cases, complete recovery usually occurs within 2 months, and recurrences are extremely rare. Chronic, active infections are refractory to therapy and carry a poor prognosis.⁶⁹ EBV infections in immunocompromised patients may be life-threatening.

CRITICAL THINKING QUESTION

16-2 Is infectious mononucleosis transmissible by the sharing of a straw?

Cytomegalovirus Infection Like EBV, Cytomegalovirus (CMV) also belongs to the herpes virus family and is endemic worldwide. The virus may be transmitted by oral, respiratory, and sexual means or by blood transfusion and organ transplantation. Patients with CMV infection may have recurrent infection or reactivation of a latent infection, as seen in herpes simplex infection, or may be reinfected with a different CMV strain. CMV infection is the most common cause of heterophile-negative IM. The diagnosis is made by demonstrating the presence of IgM antibodies to CMV or by real-time polymerase chain reaction (PCR).

Clinical Findings

In the majority of immunocompetent individuals, CMV infection is usually asymptomatic. The clinical manifestations reflect the involved organ(s). When CMV infection occurs in previously healthy individuals, the symptoms mimic EBV infection. Symptoms include fever, sore throat, splenomegaly, lymphadenopathy, and myalgia.

Laboratory Results

Morphological changes in lymphocytes are indistinguishable from those seen in EBV infection. Because of liver involvement, mild to moderate elevations of liver function tests are common.

Treatment, Clinical Course, and Prognosis

In immunocompromised individuals, CMV is a significant cause of morbidity and mortality, especially with the increase in the numbers of individuals who are immunocompromised from chemotherapy, immunosuppressive drugs, hematopoietic cell transplant, solid organ transplant, neonates, and primary immunodeficiency, including human immunodeficiency virus (HIV) infection.⁷⁶ Transmission in immunocompromised subgroups can occur as the primary infection of a previously seronegative individual, by reactivation of latent virus, or as secondary infection by an alternate viral strain.^{76,77} Because CMV infection is endemic, congenitally acquired infection is of concern and ranks as the most prevalent congenital infection worldwide.⁷⁷ In healthy patients, similar to EBV, antibiotics are usually not prescribed and the virus is transient.

Other Viral Infections Any viral infection has the potential to elicit an immune response, resulting in an absolute lymphocytosis. Lymphocytes that appear “stimulated” or reactive may be seen in CMV and infectious hepatitis, in addition to IM.

With a negative Monospot test, other viral infections should be considered.^{17,18}

Bacterial Infections Lymphocytosis in bacterial infections occurs more commonly in chronic infections and during the recovery period following acute infections. The organisms most often responsible include *Brucella species*, *Mycobacterium tuberculosis*, and spirochetes.¹⁷ Leukocytosis marked by absolute lymphocytosis is often seen in children with pertussis infection (whooping cough).^{19,78} Morphologically, pertussis lymphocytosis is characterized by mature-appearing lymphocytes, appearing similar to those seen in CLL/small lymphocytic lymphoma (SLL). Lymphocytic nuclei can also appear cleaved. The lymphocyte populations are predominantly helper T cells.⁷⁸ Prominent lymphadenopathy is notably unusual in children with pertussis or infectious lymphocytosis.

Morphological Comparisons Between Reactive Lymphocytes and Malignant Cells Malignant disorders that may be confused with reactive lymphocytoses are listed in Box 16-14. In general, the malignant cells in leukemia and lymphomas are homogenous in appearance and, therefore, are morphologically distinct from the pleomorphic reactive lymphocytes seen in benign conditions. In contrast to reactive lymphocytes, blasts have fine chromatin, prominent nucleoli, and less cytoplasm compared with reactive lymphocytes (Figs. 16-17 and 16-18). Granular blasts and prolymphocytes can also be distinguished from reactive lymphocytes using nuclear and cytoplasmic features (Figs. 16-19 and 16-20). Patients with lymphoma may have a leukemic phase with circulating malignant cells that can often be distinguished by characteristic nuclear clefts (Fig. 16-21). Malignant lymphoid cells in leukemias and lymphomas are identified as monoclonal populations when immunophenotypically analyzed. Clinically, the severe cytopenias that occur in acute leukemias are infrequent in benign lymphocytosis. With anemia or severe thrombocytopenia, the diagnosis of leukemia must be considered (see Chapter 17).

BOX 16-14 Malignant Conditions That Can Be Confused With Reactive Lymphocytosis

- **Acute Lymphocytic Leukemias**
- **Acute Myelocytic Leukemias**
- **Chronic Leukemias**
 - Chronic lymphocytic leukemia (CLL)
 - Hairy-cell leukemia
 - Lymphocytosis of large granular lymphocytes
 - Polymorphocytic leukemia
- **Leukemic Phase of Lymphoma**
 - Follicular lymphoma (typically small cleaved cell)
 - Mantle cell lymphoma
 - Other non-Hodgkin's lymphomas (e.g., large cell)
- **Miscellaneous**
 - Adult T-cell leukemia/lymphoma (ATL/L)
 - Mycosis fungoides
 - Sézary syndrome
 - Plasma cell leukemia

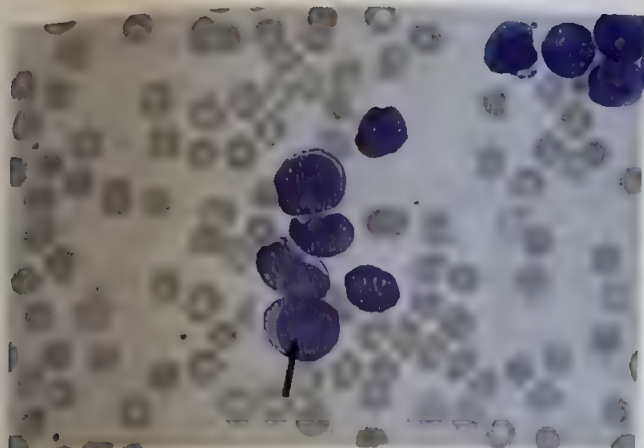


FIGURE 16-17 Acute lymphocytic leukemia. Lymphoblasts have fine chromatin with a moderate amount of cytoplasm and nucleoli. Note the prominent nucleolus in one of the lymphoblasts (arrow).

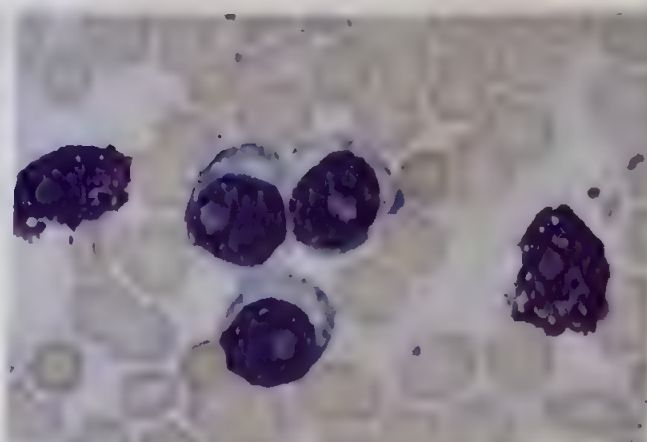


FIGURE 16-20 Prolymphocytes with moderate amounts of cytoplasm and prominent nucleoli.

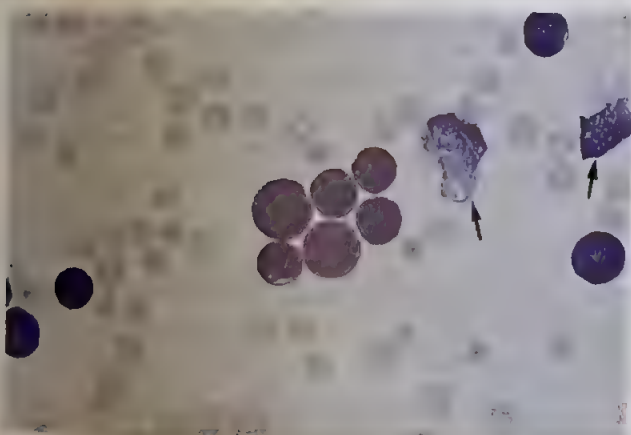


FIGURE 16-18 Acute lymphocytic leukemia. Lymphoblasts have a high N:C ratio (scant cytoplasm), fine chromatin, and indistinct nucleoli. Except for the fine chromatin, note the similarity to mature lymphocytes. These cells should not be confused with reactive lymphocytes. Note the large "smudge cells" (arrows).

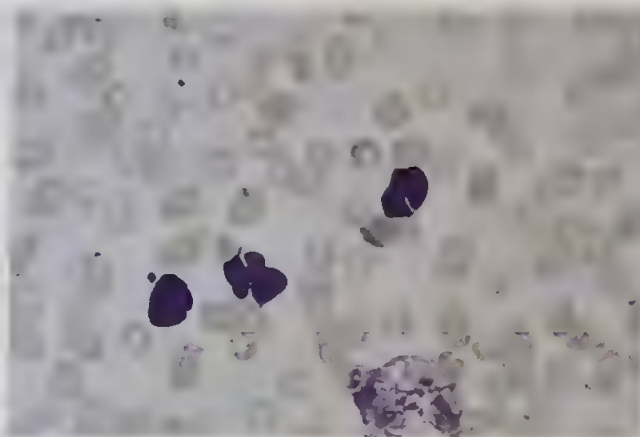


FIGURE 16-21 Circulating lymphoma cells. Note the prominent nuclear clefting (patient with follicular lymphoma)

Laboratory Testing and Results

A variety of laboratory procedures may help in the correct diagnosis of patients with an absolute lymphocytosis. CBC, serology, and microbiologic culture are often performed. Of these procedures, a CBC with differential and serological studies are the most useful.

Proper evaluation of the peripheral blood smear is crucial for the correct differential diagnosis in patients with an absolute lymphocytosis. Especially in IM, reactive lymphocytes are prominent and should easily be identified by the experienced observer. A reactive lymphocyte with abundant cytoplasm (low N:C ratio) and indented cytoplasmic borders (large arrow) is shown in Figure 16-13. The less-experienced observer may mistake these unusual-appearing reactive lymphocytes for monocytes or blasts. However, as seen in Figure 16-22, monocytes typically have linear condensation of chromatin, whereas blasts have chromatin strands that are finer and evenly dispersed.

Conversely, lymphoblasts or monoblasts may also be mistaken for reactive lymphocytes. Monoblasts have a low N:C ratio, but fine chromatin is present as well as prominent nucleoli (Fig. 16-23). The fine chromatin and prominent nucleoli (arrow) present in a lymphoblast are shown in Figure 16-17. With reactive lymphocytes, a careful review of the cell morphology

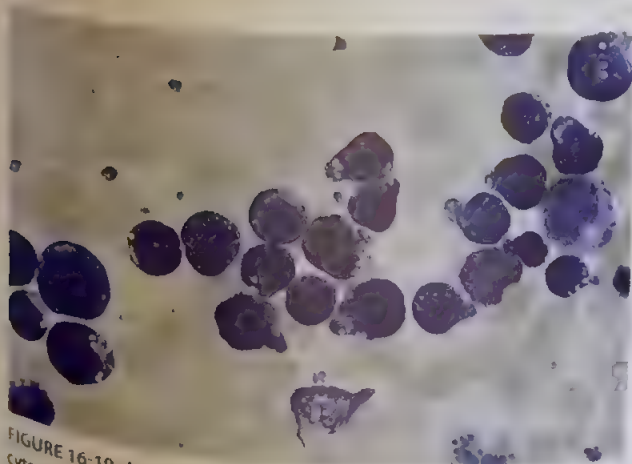


FIGURE 16-19 Acute lymphocytic leukemia. Lymphoblasts with abundant cytoplasmic vacuoles and clumped chromatin.

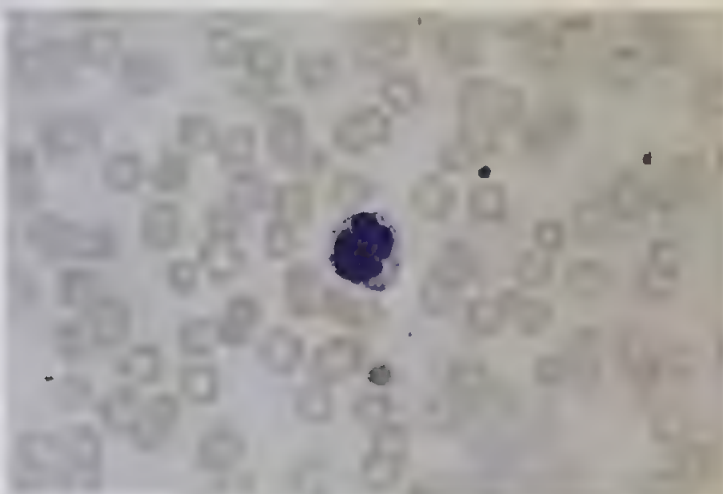


FIGURE 16-22 This monocyte has very fine granular cytoplasm, cerebriform nucleus, linear condensation of chromatin, and no nucleolus.

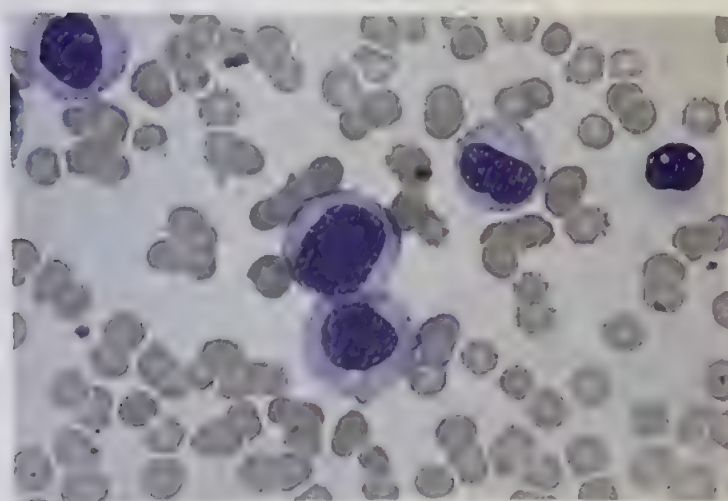


FIGURE 16-23 Acute monocytic leukemia. These monoblasts have abundant cytoplasm (low N:C ratio), fine chromatin with some chromatin clumping, and prominent nucleoli.

will reveal that, overall, the chromatin is not fine enough for the cells to be classified as blasts, and the overall morphology is variable and *not* monotonous as seen in leukemias.

Serological tests play a critical role in establishing the diagnosis in patients with an absolute lymphocytosis. In the proper clinical setting, a positive Monospot (heterophile antibody) test with reactive lymphocytes in the peripheral blood (see Fig. 16-13) is diagnostic of IM. The Monospot test is most frequently used for diagnosing IM, because the rise in titer of heterophile antibodies parallels the rise in titer of the more specific EBV antibodies. The Monospot test is much quicker, easier to use, and less expensive than measuring viral-specific antibodies.

In the first week of viral infections, IgM antibodies are formed against viral capsid antigens. During the second week, as the immunological response matures, IgG antibodies are formed. A rise in titer should be demonstrated by comparing serum obtained during acute and convalescent phases. Also, by using enzyme-linked immunosorbent assay (ELISA) techniques, antibodies to CMV and hepatitis may be measured. When results are indeterminate, viral genomes can be detected from peripheral blood using polymerase chain reaction (PCR) or in tissue using DNA probes.

If the Monospot test is initially negative, it should be repeated in 1 week if IM is suspected. Serum may be analyzed for antibodies to specific EBV antigens; some of the possible testing strategies are diagrammed in Figure 16-24. A variety of antibodies formed by humoral responses can be measured when it is necessary to further elucidate the etiology of lymphocytosis in difficult cases or in cases in which the Monospot test is negative (Table 16-8). In addition, testing for cytomegalovirus should be considered since CMV does cause similar clinical features as IM. With leukemias and lymphomas, flow cytometric immunophenotyping usually reveals a monoclonal cell population.¹⁷ As previously described in IM, B cells are infected by EBV with most of the reactive lymphocytes representing polyclonal T cells.

CRITICAL THINKING QUESTION

16-3 Could you see reactive lymphocytes on the peripheral blood smear of a patient infected with cytomegalovirus?

Lymphocytopenia

As mentioned previously, the normal percentage of lymphocytes present on examination of the peripheral smear is 20% to 40% for adult patients.¹⁸ Severe lymphocytopenia occurs when the absolute count is less than 1,000 lymphocytes/mm³ or 1.0×10^9 cells/L in adults,⁷⁹ with variances corresponding to age present in pediatric populations, consistently decreasing from birth until reaching normal adult values.⁸⁰ The disorders associated with lymphocytopenia are listed in Box 16-15; relative neutropenia and normocytic, normochromic anemia may also be present.

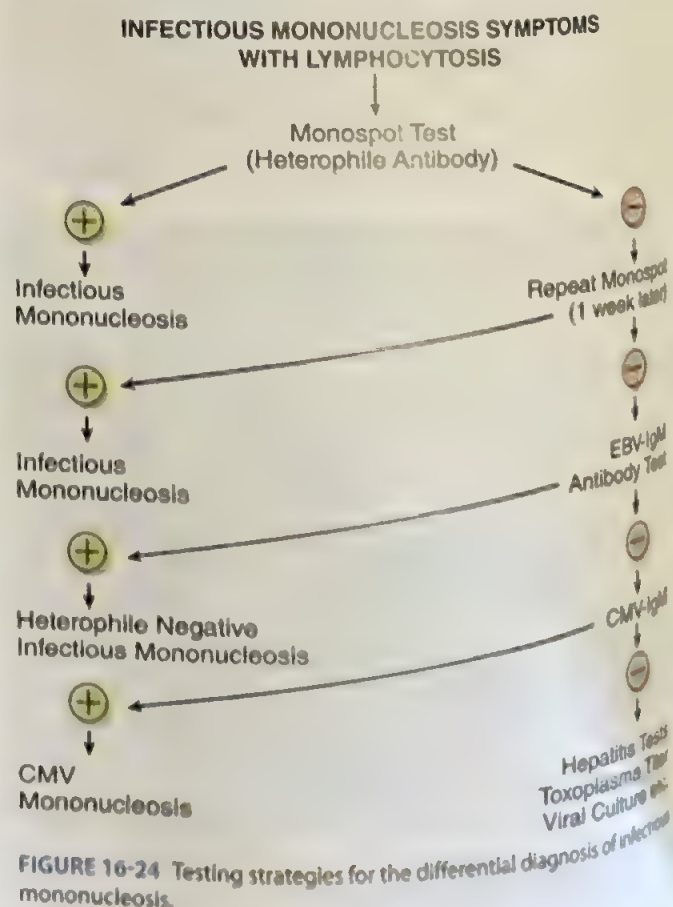


FIGURE 16-24 Testing strategies for the differential diagnosis of infectious mononucleosis.

TABLE 16-8 Summary of Antibody Tests Useful in the Differential Diagnosis of Infectious Mononucleosis

Antigen	Test Used	Description	Clinical Significance
Heterophile	Monospot	Antibodies to a variety of antigens	Appears late in the first week; detected with Monospot test; transient
EBV-VCM (IgM)	ELISA	IgM antibody to viral capsid antigen	Detectable in first week of infection; earliest detectable antibody; declines rapidly after second week
EBV-VCA (IgG)	ELISA	IgG antibody to viral capsid antigen	Detectable approximately 7 days after exposure; levels persist for life; responsible for immunity
EBNA	ELISA	Antibody to EBV nuclear antigen	Appears late in first month of infection and persists for life; may indicate past infection
EBV-EA	ELISA	Antibody to EBV early antigen complex	Seen in < 5% of normal, healthy subjects; may indicate EBV-carrier state

BOX 16-15 Causes of Secondary Reactive Lymphocytopenia (Absolute Counts $< 1.0 \times 10^9/L$ in Adults; $< 2.0 \times 10^9/L$ in Children)

- **Infections**
 - HIV
 - TB
 - Hepatitis
 - Influenza
 - Typhoid fever
 - Pneumonia
 - Babesiosis
 - Sepsis
- **Autoimmune Disorders**
 - SLE
 - Rheumatoid arthritis
 - Myasthenia gravis
- **Medical Treatment Complication**
 - Chemotherapy
 - Radiation therapy
 - Glucocorticoid
 - Anesthesia and surgery
 - Antilymphocyte globulin
 - Thoracic duct drainage
- **Systemic Diseases**
 - Sarcoidosis
 - Renal disease
 - Burns
 - Celiac disease
- **Malignant Disorders**
 - Hodgkin's lymphoma
 - Carcinoma
- **Other**
 - Congenital immunodeficiency disorders
 - Nutritional deficiencies
 - Idiopathic CD4⁺ T lymphopenia

SUMMARY CHART

- Neutrophils are capable of amoeboid movement into the tissues to engulf and destroy bacteria and fungus.
- Phagocytosis occurs in three distinct phases: migration and diapedesis; opsonization and recognition; and ingestion, killing, and digestion.
- The three modes of migration that contribute to efficient neutrophil mobilization to a site of injury are random locomotion, directional chemotaxis, and accelerated chemokinesis.
- A *shift to the left* is defined as the early release of bands and metamyelocytes from the bone marrow into circulation in response to infection or inflammation.
- An absolute cell count is determined by multiplying the percentage of cells (from the peripheral blood differential) by the total leukocyte count (from the complete blood count).
- *Neutrophilia* is an increase in the number of circulating neutrophils.
- *Neutropenia*, which can be acquired or congenital, is defined as an absolute decrease in the number of circulating neutrophils.
- In response to bacterial infection, reactive changes in neutrophil morphology can be observed in the forms of toxic granulation, Döhle bodies, and vacuolization.

SUMMARY CHART—cont'd

- Chediak–Higashi syndrome is a rare disorder of neutrophil function that is characterized by recurrent bacterial infections, partial albinism, and the presence of giant lysosomal granules in nucleated cells.
- Chronic granulomatous disease, the best understood disorder of neutrophil function, is a defect that is attributed to one of four mutations in NADPH oxidase, resulting in ineffective bacterial killing.
- Myeloperoxidase deficiency impedes phagocytic killing; patients are rarely symptomatic but can present as pseudoneutropenic using some automated differential platforms.
- In Pelger–Huët anomaly, more than 70% to 90% of neutrophils have a bilobed nucleus or no nuclear segmentation at all.
- In Alder's anomaly, prominent, dark-staining, coarse cytoplasmic granules are observed in neutrophils.
- Individuals with May–Hegglin anomaly demonstrate large, dark blue-staining cytoplasmic inclusions in granulocytes, thrombocytopenia, and giant platelets.
- *Lymphocytosis* is known as an increase in the number of circulating lymphocytes.
- *Lymphocytopenia* is a decrease in the number of circulating lymphocytes.
- The term *reactive lymphocytes* is used to describe transformed or benign lymphocytes, which usually account for less than 10% of the total lymphocytes present.
- Reactive changes in lymphocyte morphology are heterogeneous and include a low N:C ratio; round, indented, or lobulated nucleus; abundant, uneven staining cytoplasm with a round or indented cytoplasmic border; and the possible presence of nucleoli.
- Malignant cells in leukemia and lymphoma can be distinguished morphologically from reactive lymphocytes in as much as malignant cells are monotonous in appearance, similar in size and shape, as they originate from a single clone.
- Reactive changes in lymphocytes commonly accompany infectious mononucleosis, cytomegalovirus (CMV), rubella, hepatitis, and other viral infections.
- Caused by the Epstein–Barr virus (EBV), infectious mononucleosis is characterized by sore throat, fatigue, fever, headache, difficulty swallowing, and generalized malaise in teenagers and young adults.

CASE STUDY 16-1**HISTORY OF PRESENT ILLNESS**

A 3-year-old boy was admitted with a diagnosis of liver abscess after ultrasound examination. On physical examination, lymph nodes and liver were enlarged. The skin was covered with scattered areas of crusted scabs evident of an infected eczematoid rash. A skin scraping of the infected dermis revealed gram-positive cocci in clusters (*Staphylococcus*).

A review of the patient's chart showed recurrent episodes of infection since birth. Types of infection included upper and lower respiratory infections with staphylococci and enterobacteriaceae, fungal tissue abscess, and osteomyelitis. Despite these episodes of infection, he experienced normal growth and development. The family medical history was remarkable in that an older brother and a cousin (a son of a maternal aunt) had suffered similar chronic infections, and one had died of chronic pneumonia.

LABORATORY DATA

The hemoglobin level was 9 g/dL; the erythrocyte morphology was normal with slight hypochromia. The total WBC count was $33.0 \times 10^9/L$ ($28.0 \times 10^9/L$ neutrophils). The neutrophils contained moderate toxic granulation and vacuoles. The platelet count was $427 \times 10^9/L$. Concentrations of serum immunoglobulins and complement components were all moderately increased. Neutrophilia was consistently found on several occasions on retrospective review of previous laboratory data. Neutropenia was never documented. Studies

of neutrophilic migration and phagocytosis were normal. Oxidative metabolism in response to neutrophilic stimulation was completely absent. Specifically, there was no postphagocytic increase in oxygen consumption, and superoxide anion and hydrogen peroxide were not formed. Finally, neutrophils were unable to oxidize and kill *Staphylococcus* that had been phagocytized. Neutrophils from the mother were analyzed and performed at about 50% of normal capacity.

QUESTIONS

1. In reviewing the laboratory results, what can you identify about the patient?
2. Are the neutrophils working well in their role of phagocytosis? Why or why not?
3. What diagnosis is anticipated for this patient?

ANSWERS

1. The patient has anemia and neutrophilia.
2. The neutrophils migrate normally, are capable of phagocytosis, and form phagocytic vesicles. The neutrophils of these patients, however, are unable to kill the phagocytized microorganisms as they are not able to generate active oxygen metabolites (such as superoxide anion and hydrogen peroxide).
3. This patient exhibits characteristic features of the X-linked recessive form of chronic granulomatous disease of childhood, with severe and persistent infections caused by *Staphylococcus* organisms.

CASE STUDY 16-2

HISTORY OF PRESENT ILLNESS

A 19-year-old college student was seen in the student clinic with a complaint of a sore throat, a 1-week history of general malaise, and fever. She also complained of some nausea and difficulty in drinking fluids. Physical examination revealed bilateral, enlarged, firm cervical lymph nodes, mild splenomegaly, and hepatomegaly.

LABORATORY DATA

The laboratory data revealed a WBC count of $15.0 \times 10^9/L$; a hematocrit of 42%; a platelet count of $215 \times 10^9/L$, and a reticulocyte count of 2.0%. A differential count of the peripheral blood revealed 65% lymphocytes, 25% granulocytes, and 10% monocytes. The differential report noted that 36% of the lymphocytes were reactive (see Fig. 16-13). Chemistry results showed that liver enzymes and bilirubin

levels were slightly increased. No other abnormalities were noted.

After physical examination, additional laboratory work was ordered. The throat culture for streptococci was negative. A Monospot test was positive. After a period of recovery, the patient was allowed to return to school. No further problems were noted.

QUESTIONS

1. What diagnosis is anticipated for this patient?
2. What would be the diagnosis if the Monospot and EBV-VCA tests were negative?

ANSWERS

1. EBV causing infectious mononucleosis.
2. Cytomegalovirus (CMV) patients exhibit the same clinical features and laboratory findings as EBV.

REVIEW QUESTIONS

1. A patient with a bacterial infection will circulate neutrophils ready for:
 - a. The process of phagocytosis
 - b. Migration from marginating pool to circulatory pool
 - c. Release from bone marrow
 - d. Increased vitamin B₁₂
2. What are the enzymatic contents of primary (azurophilic) granules?
 - a. NADPH oxidase and hydrogen peroxide
 - b. Cytochrome b and collagenase
 - c. Myeloperoxidase and lysozyme
 - d. Alkaline phosphatase and gelatinase
3. What is the term for the directional migration toward a gradient stimulated by a chemoattractant?
 - a. Chemotaxis
 - b. Random mobility
 - c. Opsonization
 - d. Chemokinesis
4. What is the term for the marking of an invading microbe with IgG and complement to facilitate recognition?
 - a. Chemokinesis
 - b. Opsonization
 - c. Phagolysosome fusion
 - d. Signal transduction
5. Which sequence reflects the correct order for phagocytosis?
 - a. Release of cytoplasmic granules; binding of particle; ingestion; fusion of phagolysosome
 - b. Ingestion; binding of particles; fusion of phagolysosome; release of cytoplasmic granules
 - c. Binding of particle; ingestion; fusion of phagolysosome; release of cytoplasmic granules
 - d. Fusion of phagolysosome; binding of particle; release of cytoplasmic granules; ingestion
6. In oxygen-dependent killing, what is the enzyme responsible for mediating the production of active oxygen metabolites during the respiratory burst?
 - a. Serine protease
 - b. Lysozyme
 - c. Lactoferrin
 - d. NADPH oxidase
7. What are the two most important biochemical products of the respiratory burst that are involved with particle digestion during active phagocytosis?
 - a. Lactoferrin and gelatinase
 - b. Superoxide dismutase and catalase
 - c. Glutathione peroxidase and copper-zinc enzymes
 - d. Superoxide anion and hydrogen peroxide

REVIEW QUESTIONS—cont'd

8. What are the morphological characteristic(s) associated with Chediak-Higashi syndrome?
 - a. Giant lysosomal granules
 - b. Hypersegmented agranular neutrophils with vacuolization
 - c. Prominent dark-staining granules and pyknotic nuclei
 - d. Pale blue inclusions in cytoplasm of neutrophils and giant platelets
9. Myeloperoxidase deficiency causes defective:
 - a. Migration of neutrophils
 - b. Fusion of phagosomes and lysosomes
 - c. Production of hypochlorous acid
 - d. Neutrophil degranulation
10. What disease characteristic(s) is/are associated with Chediak-Higashi syndrome?
 - a. Partial albinism, recurrent infections, and mild bleeding tendencies
 - b. Granulomas, osteomyelitis, and hepatic abscesses
 - c. Hypopigmentation of skin and chronic, swollen lymph nodes
 - d. Periodic pneumonia that may result in lesions called pneumatocoles
11. Which of the following describes Pelger-Huët anomaly?
 - a. Large neutrophils and hypersegmentation of the nucleus with greater than six lobes
 - b. Dark-staining, coarse granules in cytoplasm of neutrophils, eosinophils, basophils, and monocytes
 - c. Pale blue inclusions of the cytoplasm of neutrophils and giant platelets
 - d. Hyposegmentation of the nucleus with the majority of neutrophils being bilobed or monolobed
12. Reactive lymphocytes can best be distinguished from blasts by the presence of which of the following morphological features?
 - a. Prominent nucleoli
 - b. Fine chromatin
 - c. Heterogeneous cell population
 - d. High N:C ratio
13. Which of the following antigens is detectable first by ELISA?
 - a. EBNA
 - b. EBV-VCA (IgM)
 - c. EBV-VCA (IgG)
 - d. Heterophile
14. The Epstein-Barr virus infects which of the following cells?
 - a. Helper T lymphocytes
 - b. Cytotoxic T lymphocytes
 - c. B lymphocytes
 - d. NK cells
15. In which of the following conditions are reactive lymphocytes found?
 - a. Pelger-Huët anomaly
 - b. Neutropenia
 - c. CMV
 - d. Alder-Reilly
16. What is the most frequent cause of a heterophile (Monospot) negative mononucleosis-like syndrome?
 - a. HIV
 - b. CMV
 - c. Hepatitis C
 - d. *Toxoplasma gondii*
17. Which of the following best describes absolute lymphocytosis?
 - a. Greater than 70% lymphocytes on differential
 - b. Presence of nucleoli in lymphocytes
 - c. Monoclonal population of lymphocytes
 - d. Greater than 4.0×10^9 lymphocytes per liter in an adult
18. Lymphocytosis characterized by small, mature appearing lymphocytes is demonstrated during which infection?
 - a. Epstein-Barr virus (EBV)
 - b. Cytomegalovirus (CMV)
 - c. *Bordetella pertussis*
 - d. *Staphylococcus aureus*
19. Which clinical manifestations would be unexpected in infectious mononucleosis?
 - a. Skin rash
 - b. Sore throat
 - c. Fatigue
 - d. Fever
20. Which feature best differentiates malignant lymphomas from infectious mononucleosis?
 - a. Polyclonal population
 - b. Heterogenous appearance
 - c. Genetic mutation
 - d. Cellular morphology
21. Which viral agent causes infectious mononucleosis?
 - a. Heterophile virus
 - b. Human herpes-6 virus
 - c. EBV
 - d. HIV

See answers at the back of this book.

Introduction to Leukemia and the Acute Leukemias

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CHAPTER OUTLINE

Overview of Leukemia

Incidence and Prevalence
Clinical Findings
Historical Perspectives
Etiology and Risk Factors

Acute Leukemia

Incidence
Clinical Findings
Evaluation of Morphology

Acute Myeloid Leukemia

FAB Classification
WHO Classification

Laboratory Evaluation of Acute Leukemia

Specimens
Cytochemistry
Immunological Marker Studies
Flow Cytometry
Genetic Analysis
Cytogenetics and FISH
Molecular Studies

Six Major Categories of the WHO Classification

AML with Recurrent Genetic Abnormalities
AML with Myelodysplasia-Related Changes
Therapy-Related Myeloid Neoplasms
Acute Myeloid Leukemia, Not Otherwise Specified
Myeloid Sarcoma
Myeloid Proliferations Related to Down Syndrome

Acute Lymphoblastic Leukemia/ Lymphoma (ALL/LBL)

Review of Lymphocyte Ontogeny
Clinical Findings
Morphology
Historical Classification: FAB
Classification of ALL
World Health Organization
Classification of ALL

Abnormalities

T-Lymphoblastic Leukemia/ Lymphoma
Burkitt's Leukemia/Lymphoma (Mature B-Cell ALL)
Childhood versus Adult ALL
Acute Leukemias of Ambiguous Lineage
Acute Leukemia of Ambiguous Lineage, Not Otherwise Specified

Treatment of Acute Leukemia

Summary Chart

Case Study 17-1

Case Study 17-2

Case Study 17-3

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 17-1 Define leukemia and differentiate general requirements for acute and chronic leukemia according to FAB and WHO classifications.
- 17-2 Describe characteristic morphology and cytochemical staining patterns for the subtypes of acute myeloid leukemia.
- 17-3 Assess the morphological and cytogenetic abnormalities associated with the WHO categories of acute myeloid leukemias.
- 17-4 Correlate cellular presentation of AML with diagnosis and prognosis implications.

- 17-5 Evaluate the role of genetic analysis of newly diagnosed leukemia, including the prognostic implications of the common genetic alterations seen in acute leukemias.
- 17-6 Provide the flow cytometry and genetic analysis results seen in ALL.
- 17-7 Correlate blast morphology in ALL with flow cytometry and genetic testing.
- 17-8 Analyze the morphological and cytogenetic abnormalities associated with ALL.
- 17-9 Evaluate the stages of treatment for patients with acute leukemia.

Leukemia is a malignant disease of hematopoietic tissue, characterized by replacement of normal bone marrow elements with neoplastic blood cells. These leukemic cells pass from the bone marrow into the peripheral blood, and they can also infiltrate organ and reticuloendothelial tissue, including

the spleen, liver, and lymph nodes. Leukemia can be a rapidly progressive disease characterized by the expansion of immature cells or blasts (acute leukemia) or a slowly progressive disorder characterized by an abnormal expansion of mature cells (chronic leukemia).

Today the clinical laboratory plays an important role in the diagnosis and classification of leukemia. The morphological analysis of leukemia is now augmented by cytochemical, cytogenetic, immunological, and molecular techniques. Together, these methods are used to delineate specific categories of leukemia for which distinct treatment protocols are used.

Overview of Leukemia

Leukemia can be divided into two major cell types: myeloid and lymphoid. The term “myeloid” (from *myelo*, Greek for marrow, and *eidos*, form) encompasses granulocytic, monocytic, megakaryocytic, and erythrocytic leukemias, whereas the term “lymphoid” encompasses leukemias of B-cell or T-cell origin. Each has an acute and chronic form, which leads to four major types of leukemias:

1. Acute myeloid leukemia (AML)
2. Chronic myeloid leukemia (CML)
3. Acute lymphoblastic leukemia (ALL)
4. Chronic lymphocytic leukemia (CLL)

Incidence and Prevalence

The overall incidence of leukemia in the United States is currently 14 new cases per 100,000 individuals per year. In 2021, an estimated 61,090 new cases of leukemia were diagnosed in the United States.¹ The number of new cases in 2021 for each type of leukemia are listed in Table 17-1.

Most cases of leukemia occur in older adults with a median age at diagnosis of 67 years. Currently, the ratio of adult cases to childhood cases is 12:1.¹ The most common types of leukemia in adults are acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL). Chronic leukemia, whether of lymphoid or myeloid lineage, is generally considered to be a disease of adults. CLL is extremely rare in children and is unusual before the age of 40. CML increases dramatically among individuals who are 60 years of age or older, with the most prevalent incidence in the 70- to 90-year-old range.¹ In children, leukemia is the most common cancer and the second leading cause of cancer deaths after cancers of the brain and nervous tissue. The most common form of leukemia in children is acute lymphoblastic leukemia (ALL).

The frequency of all types of leukemia is slightly higher among males than females, with approximately 58% of new

cases of leukemia diagnosed in males in 2021. In terms of race and ethnicity, leukemia rates are higher among non-Hispanic whites than among Hispanics, non-Hispanic blacks, and American Indians/Alaskan natives, and the lowest among Asian and Pacific Islanders. In the United States, an estimated 397,501 individuals are living with leukemia or are in remission.¹

Clinical Findings

The clinical and laboratory features of acute and chronic leukemia differ in several respects, as summarized in Table 17-2. Patients with acute leukemia usually present with a sudden onset of symptoms, and the disease runs a rapidly fatal course of 6 months or less, if left untreated. In contrast, patients with chronic leukemia tend to have an insidious onset and a more indolent clinical course, usually lasting 2 to 6 years. Mean survivals in both acute and chronic leukemia are improving as diagnostic sensitivities increase and patients are diagnosed at an earlier stage in their disease.

Patients with acute and chronic leukemia also show differences in their hematologic parameters. In general, bone marrow failure and its sequelae are much more prominent in the initial presentation of acute leukemia than in that of the chronic form. In acute leukemia, there is a loss of normal bone marrow function. Anemia is consistently observed in the acute leukemic patient, although its severity is variable. Thrombocytopenia is common. The white blood cell count varies; it can be markedly elevated with numerous blasts, normal, or, in some cases, even decreased. Typically, the various normal circulating white blood cells are diminished in number resulting in a neutropenia. In chronic leukemic patients, anemia is often mild at presentation. The platelet count is usually normal, but it may be increased in CML. Chronic leukemias characteristically present with elevated white blood cell counts, which are often very high ($>50,000/\mu\text{L}$).

Both acute and chronic leukemia patients can display enlargement of the spleen, liver, or lymph nodes. However, this condition is more consistently seen in patients with chronic leukemia, in whom organomegaly tends to be more prominent and can occasionally be massive.

TABLE 17-1 Number of New Cases of Each Type of Leukemia (2021)	
Type of Leukemia	Number of Cases
Acute myeloid leukemia	20,240
Acute lymphocytic leukemia	5,690
Chronic myeloid leukemia	9,110
Chronic lymphocytic leukemia	21,250

Source: The American Cancer Society. Cancer Facts and Figures 2021. Atlanta: The American Cancer Society; 2021.

TABLE 17-2 Clinical Features of Acute and Chronic Leukemia

Features	Acute Leukemia	Chronic Leukemia
Age	All ages	Adults
Clinical onset	Sudden	Insidious
Course (untreated)	<6 mo	2-6 yr
Leukemic cells	Immature	Mature
Anemia	Mild to severe	Mild
Thrombocytopenia	Mild to severe	Mild
White blood cell count	Variable	Increased
Organomegaly	Mild	Prominent

CRITICAL THINKING QUESTION**17-1** How do acute and chronic leukemia present differently?

See answers to all Critical Thinking Questions at the back of this book.

Historical Perspectives

The invention of the compound microscope in 1590 by Hans and Zacharias Janssen allowed for the initial morphological examination of blood.² Subsequently, the initial description of leukemia as a clinical entity was made by John Bennett in Scotland and Rudolf Virchow in Germany, who independently published their findings in 1845. They described a series of autopsy studies of victims of a progressive chronic disorder of unknown origin in which enlarged spleens and purulent-appearing blood were found. The blood, when examined microscopically, revealed an astounding increase in “colorless” corpuscles. Bennett initially suggested that the marked increase in white blood cells was the result of an inflammatory process. Virchow chose the term *Weisses Blut* (white blood), which was later translated into Greek as *leukemia*. He proposed that leukemia was caused by a neoplastic proliferation of white blood cells.² The ensuing debate between Bennett and Virchow continued for several years, but eventually even Bennett rejected inflammation as the etiology of leukemia.

Virchow continued his study of leukemia and defined two groups characterized predominantly by either splenic or nodal involvement. Today, these are recognized as chronic myelogenous leukemia and chronic lymphocytic leukemia, respectively.

In 1857, the pathologist Nikolaus Friedreich introduced the first classification of leukemia. Further classification was made possible in 1877 by Paul Ehrlich's discovery of a triacid stain that permitted the morphological characterization of blood cells under the microscope.² Examination of the morphology enabled investigators to show that acute leukemia was associated with immature cells, whereas chronic leukemia was associated with mature, well differentiated cells. At the turn of the century, Naegeli described the myeloblast and divided the acute leukemia into myeloblastic and lymphoblastic forms. A decade later, Shilling described a monoblastic (blasts with the morphological features of immature monocytes) variant of the myeloblastic form. Thus, the main morphological variants of acute and chronic leukemia were well established by 1930. Since that time, classification of leukemia has been refined, and clinically distinct subgroups have been characterized.

Today, the clinical laboratory plays an important role in the diagnosis and classification of leukemia. The morphological analysis of leukemia is now augmented by cytochemical, cytogenetic, immunological, and molecular techniques. Together, these methods are used to delineate specific categories of leukemia for which distinct treatment protocols are used.

Certainly, the most significant application of the biological understanding of leukemia has been the improvement in outcome as a result of continued advances in treatment. Complex therapeutic protocols with multiple cytotoxic drugs and

radiation, as well as more “targeted” and less toxic approaches such as tyrosine kinase inhibitors and protease inhibitors, are used. Bone marrow and stem cell transplantation have improved the survival of many patients with leukemia, especially those with acute forms. The effectiveness of treatment is most notable for children with acute lymphoblastic leukemia (ALL). Before the 1960s, childhood ALL was universally fatal, but with modern treatment regimens, the survival rates have dramatically improved over the past 30 years. The majority of children with ALL younger than 15 years of age are now cured.³ The survival rates, however, are not as impressive for adults with ALL and AML. For example, ALL patients older than 60 years of age have 5-year survival rates of approximately 20%.⁴ Similarly, AML patients older than 60 show poor survival rates in most instances.⁵

Etiology and Risk Factors

The origin of leukemia at the genetic level appears, in most cases, to be related to mutation and altered expression of **oncogenes** and tumor suppressor genes. Most oncogenes regulate cell proliferation and differentiation. Abnormal oncogene or tumor suppressor gene expression induced by translocation and genetic fusion, or mutation often results in unregulated cellular proliferation. Although the events that lead to this are not entirely understood, a number of host and environmental factors have been identified that are associated with increased risk of leukemia transformation. The epidemiological aspects of acute leukemia have been reviewed,⁶ and the host and environmental factors associated with increased risk of leukemia are summarized in Box 17-1.

Acute Leukemia**Incidence**

Acute leukemia occurs in individuals of all ages, but ALL is more common in children and AML is more common in adults. Seventy-four percent of childhood leukemias are classified as ALL, whereas nearly 80% of AML cases occur in adults.¹

The incidence of acute leukemia increases exponentially with age, and the median age at diagnosis of acute myeloid leukemia (AML) in the United States is currently 68 years.¹ Males have a slightly increased incidence compared with females. In 2021, there were a total estimated number of 20,240 new cases of AML and 5,690 new cases of ALL in the United States.¹

Clinical Findings

The majority of patients with acute leukemia display clinically abrupt onset of signs and symptoms of only a few weeks' duration. Patients often seek medical attention because of weakness, bleeding abnormalities, or flu-like symptoms. These abnormalities reflect the failure of the bone marrow to produce adequate numbers of normal cells and are caused by the proliferation and accumulation of leukemic cells in the marrow. Leukemic replacement eventually results in marrow failure and the resultant life-threatening complications of anemia, thrombocytopenia, granulocytopenia, and their sequelae. Anemia, the most consistent presenting feature,

BOX 17-1 Host and Environmental Factors Associated with an Increased Risk of Leukemia

Inherited Predisposing Syndromes

- Li Fraumeni Syndrome
- Neurofibromatosis Type 1
- Noonan Syndrome
- Bloom Syndrome
- Dyskeratosis Congenita

Bone Marrow Failure Syndromes

- Fanconi Anemia
- Diamond Blackfan Anemia
- Shwachman Diamond Syndrome
- Amegakaryocytic Thrombocytopenia
- Thrombocytopenia With Absent Radial Syndrome
- Severe Congenital Neutropenia

Abnormal Chromosomal Number

- Down Syndrome
- Klinefelter's Syndrome
- Monosomy 7

Immunodeficiency Syndromes

- Wiskott-Aldrich Syndrome
- Bruton Agammaglobulinemia
- Ataxia Telangiectasia

Chronic Marrow Dysfunction

- Myelodysplastic Syndrome
- Myeloproliferative Neoplasms

Environmental Factors

- Ionizing Radiation
- Chemotherapy (alkylating agents, platinum derivatives and topoisomerase II inhibitors)

Adapted from Tebbi, CK. Etiology of Acute Leukemia: A Review. *Cancers*. 2021;13(2256). <https://www.mdpi.com/journal/cancers>.

is associated with fatigue, malaise, pallor, and shortness of breath. Hemorrhagic complications related to thrombocytopenia may be mild and restricted to easy bruising, petechiae, and mucosal bleeding, or they may be more severe, involving gastrointestinal tract, genitourinary tract, or central nervous system hemorrhage. Infections result from severe granulocytopenia. Bacterial infections are common (e.g., *Staphylococcus*, *Pseudomonas*, *Escherichia coli*, and *Klebsiella*), but fungal infections also occur (e.g., *Candida* and *Aspergillus*). Viral infections are less frequent.

Infiltration of other tissues, especially organs that play a role in fetal hematopoiesis, is often manifested by hepatosplenomegaly or lymphadenopathy, particularly in ALL and the acute monoblastic leukemia, a subtype of AML. A myeloid or granulocytic sarcoma is defined as a tumor mass of myeloid cells outside of the marrow and may be the first evidence of AML. Gingival hypertrophy and oral lesions are primarily seen in acute monoblastic leukemia. A mediastinal mass resulting from thymic involvement is a hallmark of

precursor T-ALL. Bone or joint pain, caused by pressure of the expanding leukemic cell population in the marrow cavity, commonly accompanies the acute leukemias. Leukemic infiltration of the central nervous system, an ominous feature infrequently observed at initial presentation, is associated with signs and symptoms of increased intracranial pressure (nausea, vomiting, headache, papilledema) or cranial nerve palsies. These clinical features and their relationship to pathophysiology are summarized in Table 17-3.

Once the diagnosis of leukemia is suggested, a bone marrow aspirate and biopsy are obtained. Morphological examination of the bone marrow is usually required to establish the diagnosis. In addition, a series of laboratory tests help classify the disease, determine prognosis, and guide therapy. The distinction between AML and ALL is particularly important and is outlined in Table 17-4.

CRITICAL THINKING QUESTION

17-2 What CBC findings would you likely see in patients with acute leukemia?

Evaluation of Morphology

Cellular morphology is evaluated on a Romanowsky (Wright-Giemsa)-stained blood or bone marrow smear in carefully chosen areas where cells are not distorted by overcrowding. The experienced morphologist will often be able to identify blasts and classify the leukemic cell type as myeloid or lymphoid; however, additional testing is always necessary to confirm the diagnosis.

Several morphological features are helpful in distinguishing lymphoblasts from myeloblasts (Table 17-5). These include the size of the blast, amount of cytoplasm, nuclear chromatin pattern, and the presence of nucleoli. The typical myeloblast (Fig. 17-1) is a large cell (15 to 20 μm in diameter)

TABLE 17-3 Clinical Findings in Acute Leukemia

Pathogenesis	Clinical Manifestations
Bone Marrow Failure	
Anemia	Fatigue, malaise, pallor
Thrombocytopenia	Bruising, bleeding
Granulocytopenia	Fever, infections
Organ Infiltration	
Marrow expansion	Bone or joint pain
Spleen	Splenomegaly
Liver	Hepatomegaly
Lymph nodes	Lymphadenopathy
Central nervous system	Neurological symptoms
Gums, mouth	Gingival hypertrophy, oral lesions

TABLE 17-4 Comparison of Acute Myeloid and Acute Lymphoblastic Leukemia

Factor	AML	ALL
Age	Common in adults, rare in children	Common in children, rare in adults
Blood	Anemia, neutropenia, thrombocytopenia; myeloblasts and promyelocytes	Anemia, neutropenia, thrombocytopenia; lymphoblasts and prolymphocytes
Morphology	Medium-to-large blasts, more cytoplasm than lymphoblasts, cytoplasmic granules, Auer rods; fine nuclear chromatin and distinct nucleoli	Small or medium blasts, scarce cytoplasm, no granules; fine nuclear chromatin and indistinct nucleoli
Cytochemistry	Positive peroxidase and Sudan black B	Negative peroxidase and Sudan black B
Extramedullary and focal disease	Common in spleen and liver; less common in lymph nodes and CNS	Common in lymph nodes, spleen, liver, CNS, and gonads

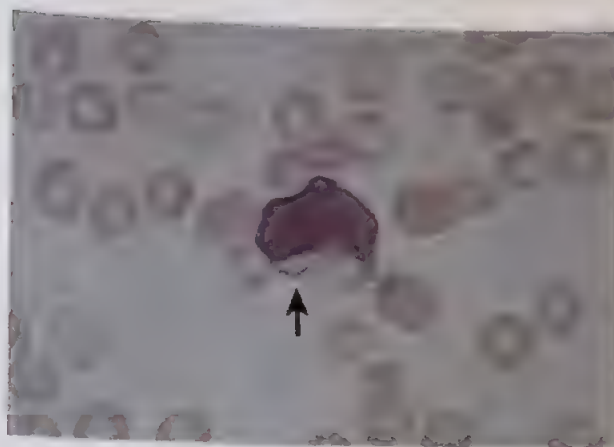
TABLE 17-5 Morphological Features of Blasts in Acute Myeloid and Acute Lymphoblastic Leukemias

Feature	AML	ALL
Blast Nuclei	Usually larger, finely dispersed chromatin, variable nucleoli	Variable size small to medium, fine to more mature chromatin, may or not have nucleoli
Cytoplasm	Moderately abundant, fine small granules often present, may see Auer rods	Usually scant, coarse granules sometimes present (~7%)
Background Marrow	May see a dysplastic background	Usually not dysplastic

Source: From Kjeldsberg, CR, editor. *Practical Diagnosis of Hematologic Disorders*. 2nd ed. Chicago: ASCP Press; 1995, p 381, with permission

with a moderate amount of cytoplasm. Its nucleus has a fine, reticulated chromatin pattern, and multiple distinct nucleoli are often present. The typical lymphoblast (Fig. 17-2) is a smaller cell with scant cytoplasm. The nuclear chromatin often appears denser than in the myeloblast, and nucleoli are usually indistinct when present.⁷ For a review of morphological descriptions of the blast stage, see Chapter 1.

Granulocytic differentiation is suggested by the presence of azurophilic granules. A very helpful morphological feature is the Auer rod, the presence of which excludes ALL. Auer rods are cytoplasmic inclusions that result from an

**FIGURE 17-1** Myeloblast (note the Auer rod).**FIGURE 17-2** Lymphoblasts (peripheral blood).

abnormal fusion of primary granules and are pathognomonic for a myeloproliferative process, particularly AML. On Romanowsky-stained smears, Auer rods appear as pink- or purple-staining rods or splinter-shaped inclusions (see Fig. 17-1). They are present in up to 60% to 70% of patients with AML,⁸ but it may take a long, careful review of the blood or marrow smear to find them; given their diagnostic importance, this search is well worth the effort. In acute promyelocytic leukemia, Auer rods are easy to find, some cells having “bundles” of cigar-shaped rods.

▶ ADVANCED CONTENT

Occasionally, patients are diagnosed with a recognizable myelodysplastic syndrome. The myelodysplastic syndromes (MDS) are more common in patients older than 50 years of age and are associated with unexplained and persistent anemia, leukopenia, and thrombocytopenia, alone or in combination. These peripheral cytopenias are typically associated with a hypercellular bone marrow. Progression of MDS into acute leukemia occurs in 30% of patients.⁹ The myelodysplastic syndromes are discussed in more detail in Chapter 20.

Acute Myeloid Leukemia

FAB Classification

In 1976, a group of French, American, and British hematologists, prompted by the need for uniform nomenclature and classification of acute leukemia, proposed a morphological classification scheme for leukemia.¹⁰ This scheme, the French–American–British Classification, better known as the FAB Classification, proved to be useful in standardizing the morphological classification of both acute myeloid and lymphoid leukemias. The FAB groups and corresponding WHO subtypes¹¹ are summarized in Table 17–6. In the years since it was first introduced, cytogenetic and molecular testing have allowed more detailed mapping of acute myeloid leukemia. The new standard for subtyping AML is the WHO Classification, which incorporates the new testing methodologies. However, the FAB Classification remains relevant and classifies the not otherwise specified (NOS) category of AML.

The FAB classification of AMLs is divided into the following groups:

- M0: Myeloid with minimal differentiation
- M1: Myeloid without maturation
- M2: Myeloid with maturation
- M3: Promyelocytic; M3m: promyelocytic microgranular variant
- M4: Myelomonocytic; M4Eo: myelomonocytic with abnormal eosinophilia
- M5: Monocytic (a) poorly differentiated and (b) well differentiated
- M6: Erythroid
- M7: Megakaryoblastic

TABLE 17-6 Classification of Acute Myeloid Leukemia

Type of Leukemia	Abbreviation	FAB*	Alternate Names
AML with minimal differentiation		M0	
AML without maturation		M1	
AML with maturation		M2	
Acute promyelocytic leukemia	APL	M3	Hypergranular promyelocytic
Acute myelomonocytic leukemia	AMML	M4	Naegeli-type leukemia
Acute monocytic leukemia	AMoL	M5	Schilling-type leukemia
Erythroleukemia	AEL	M6	Di Guglielmo's syndrome, erythremic myelosis
Acute megakaryoblastic leukemia	AMegL	M7	

*French–American–British classification of acute leukemia.

WHO Classification

Advances in the understanding of the molecular/genetic features of hematopoietic malignancies have led to the new WHO classification, which seeks to incorporate this new information. Most pathologists and hematologists/oncologists have adopted the WHO terminology in their routine practice. Importantly, however, the FAB classification remains relevant because it is incorporated into the WHO category of “AML not otherwise categorized,” and many clinicians and researchers continue to reference the FAB system. In addition, archived material and several older databases use the FAB criteria. An important distinction to keep in mind is the blast count in the blood or bone marrow for the diagnosis of AML is 20% in the WHO classification and 30% in the FAB classification. The exception in the WHO classification is a subset of AML with recurrent genetic abnormalities, which may have a lower blast count (see AML with Recurrent Genetic Abnormalities section later). In the WHO classification, recurring genetic abnormalities of the leukemic cells must be evaluated. Cytogenetic studies including karyotype and FISH analysis should be initially performed and at regular intervals to monitor residual disease. Molecular techniques such as next generation sequencing and RT-PCR have become the standard of care and provide diagnostic and prognostic information as well as identifying therapeutic targets in the leukemic cells.

The WHO classification incorporates six major categories in its classification of acute myeloid leukemias:¹³

1. Acute myeloid leukemias with recurrent genetic abnormalities
2. Acute myeloid leukemia with myelodysplasia-related changes
3. Therapy-related myeloid neoplasms
4. Acute myeloid leukemia not otherwise specified (AML, NOS).
5. Myeloid sarcoma
6. Myeloid proliferations associated with Down syndrome

A more detailed description of the WHO classification with a listing of disorders or subclasses under each major category is presented later in the chapter.

CRITICAL THINKING QUESTION

- 17-3** What is the percentage of blasts required for the diagnosis of AML according to the FAB and WHO classification systems?

Laboratory Testing of Acute Leukemia

Although a diagnosis of acute leukemia may be established by a peripheral blood examination, well-prepared smears of bone marrow aspirate material are still the specimen of choice for the classification of leukemia.

A systematic approach to the classification of leukemia begins with a review of cell morphology, providing important clues leading to a cell lineage diagnosis. For example, the presence of Auer rods in blasts is most consistent with

AML. Morphological evaluation should be followed by immunological cell marker studies and, in some cases, cytochemical staining. Immunological analysis is routinely done on new acute leukemia cases using flow cytometry, which allows determination of blast immunophenotype. There are many useful flow markers that help determine subclassification, including cytoplasmic myeloperoxidase present in some AML cases, the presence of CD19 and cytoplasmic CD22 and CD79a in B lymphoblastic leukemia, surface or cytoplasmic CD3 in T lymphoblastic leukemia, and nuclear enzyme terminal deoxynucleotidyl transferase (TdT) present in most cases of lymphoblastic leukemia and occasionally expressed in AML.

Cytochemical stains can be useful when flow cytometry analysis does not demonstrate a lineage-specific phenotype and can help define granulocytic and monocytic differentiation. When positive, cytochemical studies permit subclassification of most cases of AML Not Otherwise Specified (NOS) and help exclude ALL.

Increasingly important are laboratory studies that help identify genetic abnormalities in the malignant blasts, particularly in classifying acute myeloid leukemias and B lymphoblastic leukemias with recurrent genetic abnormalities. Such laboratory studies include conventional karyotyping, fluorescent in situ hybridization (FISH), and molecular genetic studies. FISH studies and chromosome analysis can help classify acute leukemia with recurrent genetic abnormalities and acute leukemia with myelodysplasia-related changes. In addition, they can provide prognostic information. Molecular diagnostic studies play an important and expanding role in primary diagnosis, target therapy, and detection of minimal residual disease.

The purpose and principles of these laboratory methods and detailed procedures are outlined in Chapters 34 and 35.

CRITICAL THINKING QUESTIONS

- 17-4** When acute leukemia is suspected, what additional testing should be done to confirm the diagnosis and classify the type of acute leukemia?

Specimens

Care must be taken to ensure that an adequate specimen is obtained and that it is properly handled. Lack of technical excellence, such as an inadequate or improperly handled specimen, may obscure or complicate an otherwise straightforward diagnosis.

Ethylene diaminetetraacetic acid (EDTA) (purple top) tube is the specimen of choice for most hematology testing. However, the anticoagulant EDTA can cause subtle morphological artifacts of nucleated cells and platelets. A specimen left in EDTA for longer than 30 minutes may show artifactual vacuolation of monocytes and neutrophils, nuclear shape changes and swelling, as well as degranulation of platelets.¹² These possible alterations should be kept in mind if only a routine EDTA-anticoagulated peripheral blood smear is available for

review. A smear made from a nonanticoagulated fingerstick will not show these abnormalities.

As discussed in Chapter 3, a bone marrow aspirate is often collected for confirmation testing for leukemias. During the bone marrow procedure, the aspirate is collected first and smears are made immediately to avoid clotting. As the aspirate smears are pulled, the presence of bone marrow spicules should be present to confirm the sample is indeed bone marrow. If spicules are not present, another aspiration may be necessary. For cytogenetic studies and FISH analysis, the marrow aspirate is anticoagulated by aspirating directly into a syringe coated with heparin. For flow cytometry analysis, specimens can be collected in either an EDTA tube or a heparin-coated tube. An EDTA tube is preferred; however, as a peripheral blood smear and CBC can be done on the same sample on peripheral blood, aspirate smears and cytochemical stains can be done from the same bone marrow sample. After sufficient aspirate material is collected, a bone marrow core biopsy is obtained. While smears from a bone marrow aspirate are much more common, a smear (touch preparation) from a biopsy core can help create a smear when aspirates are difficult or a dry tap occurs. Care should be taken to ensure that the biopsy has an adequate amount of bone marrow. A biopsy consisting only of periosteum and cortical bone is useless and inadequate. The biopsy is gently rolled between two glass slides to make touch preps.

Cytochemistry

Special stains have been used to identify chemical components of cells such as enzymes or lipids. Although used less often today, these cytochemical stains are still an important aid in the classification of acute leukemia because they identify cellular components that are associated with specific cell lines. When any of these stains are positive, even in a relatively small percentage of blasts, lymphoid origin is ruled out, with rare exceptions. Cytochemical stains not only help distinguish between ALL and AML but are also used to subclassify AML NOS.

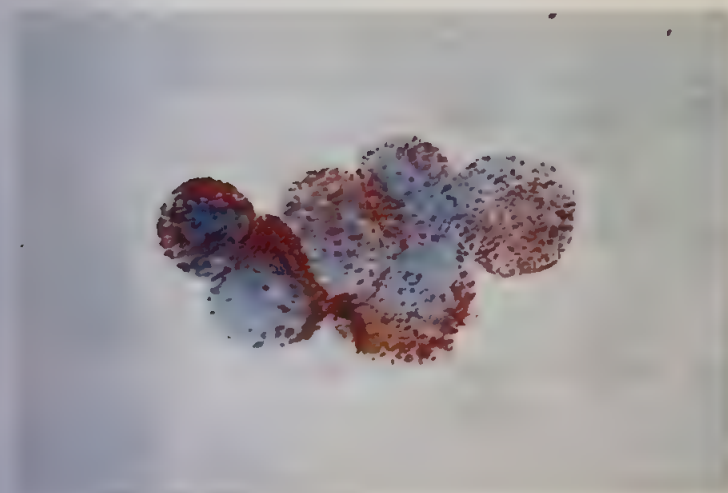
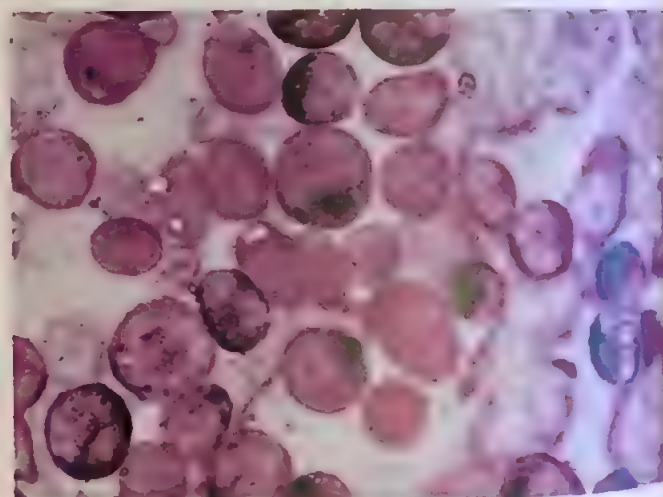
The cytochemical reactions are performed by applying staining techniques to peripheral blood smears, bone marrow smears, or touch preparations. Fresh preparations are preferred, especially for enzyme reactions. It is the leukemic cell population whose identity (cell lineage) is in question; therefore, a positive reaction is determined by finding positive staining in the leukemic blasts rather than in mature cells. The cytochemical reactions that are useful in the classification of acute leukemia are summarized in Table 17-7.

Myeloperoxidase

Myeloperoxidase (MPO) is an enzymatic stain that stains the peroxidase present in the primary granules of myeloid cells (Fig. 17-3). These granules first appear in the late blast/early promyelocyte stages and remain through cell maturation. Monocytes have variable staining with peroxidase and are most often only weakly positive. This enzyme is not present in lymphocytes or their precursors and is, therefore, useful in differentiating AML from ALL. It is more specific for granulocytic differentiation than the Sudan black B stain.^{7,13}

TABLE 17-7 Summary of Cytochemical Reactions Useful in Diagnosing Acute Myeloid Leukemia

Special Stain	Site of Action	Cells Stained	Comment
Myeloperoxidase	Mainly primary granules; Auer rods	Late myeloblasts, granulocytes; monocytes less intensely	Valuable in that the primary granules are not always visible; separates AML (+ blasts) from ALL (– blasts)
Sudan black B	Phospholipids: sterols, neutral fats	Late myeloblasts, granulocytes; monocytes less intensely	Parallels peroxidase, but smears do not need to be fresh
Specific esterase (Naphthol AS-D chloroacetate)	Cytoplasm	Neutrophilic granulocytes; mast cells	Parallels peroxidase, but less sensitive; useful on paraffin- embedded tissues
Nonspecific esterase Alpha-naphthyl acetate (ANAE) and butyrate	Cytoplasm	Monocytes; focal staining in T cells; ANAE also + in megakaryocytes	Useful for determining degree of monocytic differentiation; separates mono (+) from myelo (–) blasts
Periodic acid–Schiff	Glycogen and related substances	Lymphocytes, granulocytes, megakaryocytes	Helpful in supporting diagnosis of erythroleukemia

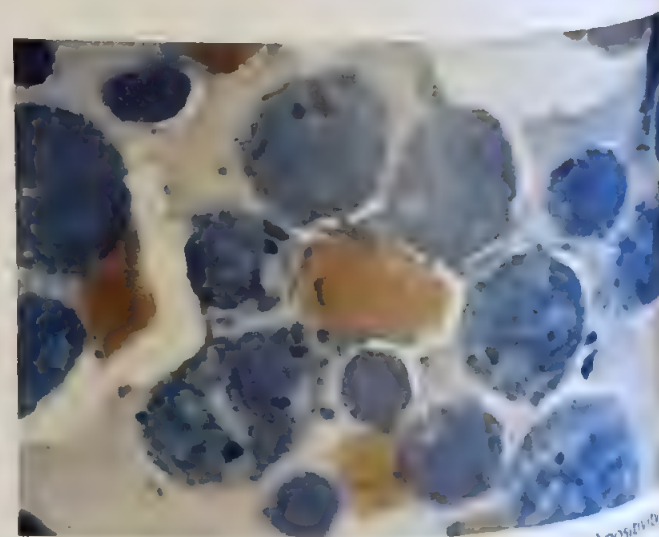
**FIGURE 17-3** Myeloperoxidase positivity in acute promyelocytic leukemia.**FIGURE 17-4** Sudan black B positivity in acute myeloblastic leukemia (AML), M2.**Sudan Black B**

Phospholipids, neutral fats, and sterols are stained by Sudan black B (SBB) (Fig. 17-4). Phospholipids occur both in primary and secondary granules of granulocytic cells and, to a lesser extent, in monocytic lysosomal granules.

SBB is the most sensitive stain for granulocytic precursors, with a staining pattern that generally parallels the myeloperoxidase stain. As with the peroxidase stain, SBB is used to differentiate AML from ALL. Positivity seldom occurs in lymphoid cells; SBB is less specific than MPO.^{7,13} SBB stain, whose reactivity does not diminish with time, is particularly useful for specimens that are not fresh.

Specific Esterase (Naphthol AS-D Chloroacetate)

The specific esterase stain (Fig. 17-5), commonly referred to as chloroacetate esterase (CAE), roughly parallels the peroxidase and SBB stains, although it is not as sensitive. It is negative in eosinophils and monocytes but positive in neutrophils, basophils, mast cells, and their precursors.

**FIGURE 17-5** Specific esterase (naphthol AS-D chloroacetate) positivity in AML, M2.

CAE, in the presence of diazonium salt, is enzymatically hydrolyzed, freeing naphthol compounds and enzymatically staining them a bright, granular red color. It is useful in demonstrating myeloid differentiation and can be done on paraffin-embedded tissue sections^{7,13} (tissue must not be acid decalcified).

Nonspecific Esterase (Alpha-Naphthyl Acetate or Butyrate)

The nonspecific esterase (NSE) stain (Fig. 17-6) is used to identify monocytes and macrophages. It is diffusely positive in these cells and negative in granulocytic cells. Lymphoid cells are negative, except for T lymphocytes, which can demonstrate a focal dotlike cytoplasmic staining pattern.^{7,13}

Periodic Acid-Schiff

The periodic acid-Schiff (PAS) reaction stains glycogen and related compounds. Strong PAS positivity may be present in erythroblasts in erythroleukemia, a potentially helpful feature for differentiating erythroleukemia from pernicious anemia (PA), in which the PAS reaction is negative in PA except in rare cases. Normoblasts may also be positive in iron deficiency, thalassemia, severe hemolytic anemias, and some of the myelodysplastic syndromes. The PAS reaction is not very useful for characterizing acute leukemia, and it is no longer commonly used for classification. The typical block positivity (Fig. 17-7) associated with lymphoblastic leukemia may also occur in acute myeloid leukemia, especially the acute monocytic type and erythroleukemia.¹³ The PAS stain, however, does not reliably distinguish AML from ALL, and typically other methods are employed to make clinically relevant distinctions.

Immunological Marker Studies

A number of immunological methods are indispensable to the diagnosis and classification of acute leukemia. Antibodies detect markers associated with cell lineage (lymphoid vs. myeloid) and maturation stage.

In clinical practice, there are essentially two techniques used to detect antigens either on the blast cell surface or within the blast cytoplasm: flow cytometry and immunohistochemistry. Today, flow cytometry is essential in the diagnosis of

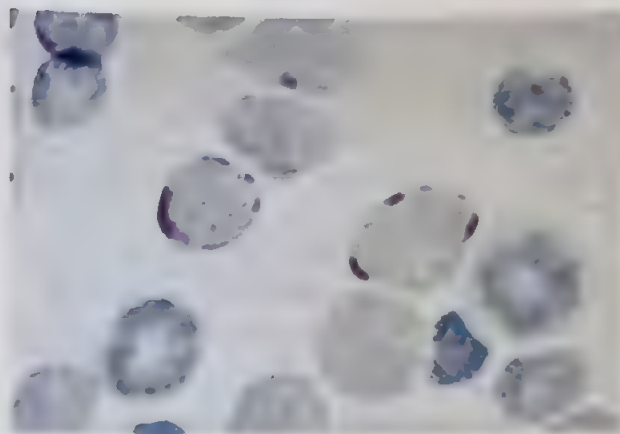


FIGURE 17-7 Periodic acid-Schiff positivity in acute lymphoblastic leukemia (ALL). Note the "block" staining pattern.

both acute and chronic leukemias. It allows the simultaneous immunophenotyping of a mixed population of cells where aberrant expression or loss of antigens can be analyzed on specific populations. In addition, it allows for evaluation of lineage specific markers that can help classify blasts as myeloid or lymphoid. Flow cytometry analysis requires a cell suspension and is best done on peripheral blood or bone marrow aspirate. When a sample for flow cytometry is not available, immunohistochemistry can be done to help evaluate the blast lineage. Immunohistochemistry is typically done on paraffin sections of the core biopsy and clot section made from the aspirate, or on paraffin sections of another tissue site biopsy material. The following paragraphs briefly introduce flow cytometry and the immunological cell marker. Their utility in the evaluation of acute leukemia is discussed in the individual sections on AML and ALL.

Flow Cytometry

Flow cytometry analysis is replacing conventional cytochemical methods for lineage determination such as AML from ALL, particularly when the leukemic cells are poorly differentiated or negative and indistinct with cytochemical stains, such as in the case of AML M0. It requires a cell suspension, typically from bone marrow aspirates or peripheral blood. It is important to have fresh specimens with viable cells; nonviable cells lead to nonspecific staining, which may make interpretation impossible. An immunofluorescent method (direct or indirect) is used to stain the cells, and a flow cytometer is used to analyze them. (See Chapter 34 for a review of flow cytometry.)

Flow cytometry allows the rapid evaluation of single cell makers on cell populations that allow their classification as myeloid cells or lymphoid cells. Cell markers are proteins on the cell membrane or cytoplasm that can be detected using flow cytometry and immunohistochemistry. The common hematology-related markers are listed in Table 17-8.

Different stages of maturation express different proteins. Some proteins are present early in development, whereas others do not appear until much later. Still other proteins may appear, disappear, and then reappear at a later stage of



FIGURE 17-6 Nonspecific esterase (alpha-naphthyl butyrate) positivity in acute monocytic leukemia (M5).

TABLE 17-8 Monoclonal Antibodies Used for Study of Leukemia and Lymphoma

Cluster Designation	Major Hematopoietic Reactivity	Cluster Designation	Major Hematopoietic Reactivity
CD1a	Thymic and Langerhans' cells	CD33	Myeloid progenitors
CD2	E-Rosette-forming T cells	CD34	Hematopoietic progenitor-stem cells
CD3	Mature T cells	CD38	Plasma cells, ALL, AML
CD4	Helper-inducer T-cell subset	CD41	Plts and megakaryocytes (GPIIb/IIIa), AML M7
CD5	Pan-T and some B cells	CD42b	Plts and megakaryocytes (GPIb), AML M7
CD7	Pan-T, early thymocytes	CD43	T cells, myeloid cells
CD8	Suppressor-cytotoxic T-cell subset	CD45	Leukocytes
CD10	B-cell pre, some thymocytes, grans and ALL	CD56	NK cells, T-cell subset
CD11b	Monos and grans, C3bi receptor	CD57	T cells, NK cells
CD11c	Monos, myeloid precursors, HCL	CD61	Plts, AML (M7)
CD13	Most grans, minority of monos, and other	CD64	Monocytes, early myeloid cells
CD14	Monos, minority of grans, and DRC	CD79a	B cells, ALL
CD15	Myeloid cells, RS	CD103	Intraepithelial lymphocytes, HCL
CD19	B cells, early B-cell precursors	CD117	AML, mast cells, early erythroid precursors
CD20	B cells, midstage B-cell precursors	CD138	Plasma cells
CD21	C3d receptor on B cells and DRC	MPO	Granulocytes, AML
CD22	B cells, ALL, HCL	PAX-5	B cells and hematogones
CD23	B cells, EBV-transformed B lymphoblasts	TdT	Hematogones, ALL
CD25	IL-2 receptor on T cells and other, HCL, NHL	HLA-DR	B cells, monocytes, macrophages, precursor T cells
CD30	Activated B and T cells, RS, ALCL		

CD = cluster designation; B cells = B lymphocyte; T cells = T lymphocyte; Pan = reactivity with many leukocyte populations; ALL = acute lymphoblastic leukemia; AML = acute myeloid leukemia; DRC = dendritic reticulum cells; grans = granulocytes; monos = monocytes; Plts = platelets; RS = Reed Sternberg cells; HCL = hairy cell leukemia; ALCL = anaplastic large cell lymphoma; NK = natural killer cells.

development. This unique expression of proteins enables the diagnostician to use these proteins as markers of both cell lineage and maturation stage. A schematic representation of surface antigen expression in normal myeloid differentiation is shown in Figure 17-8.

When surface marker analysis is performed to distinguish AML from ALL by flow cytometry, it is important to choose a panel of markers that includes antibodies to several myeloid-associated antigens, B-cell antigens, and T-cell antigens. Myelomonocytic antigens include CD13, CD14

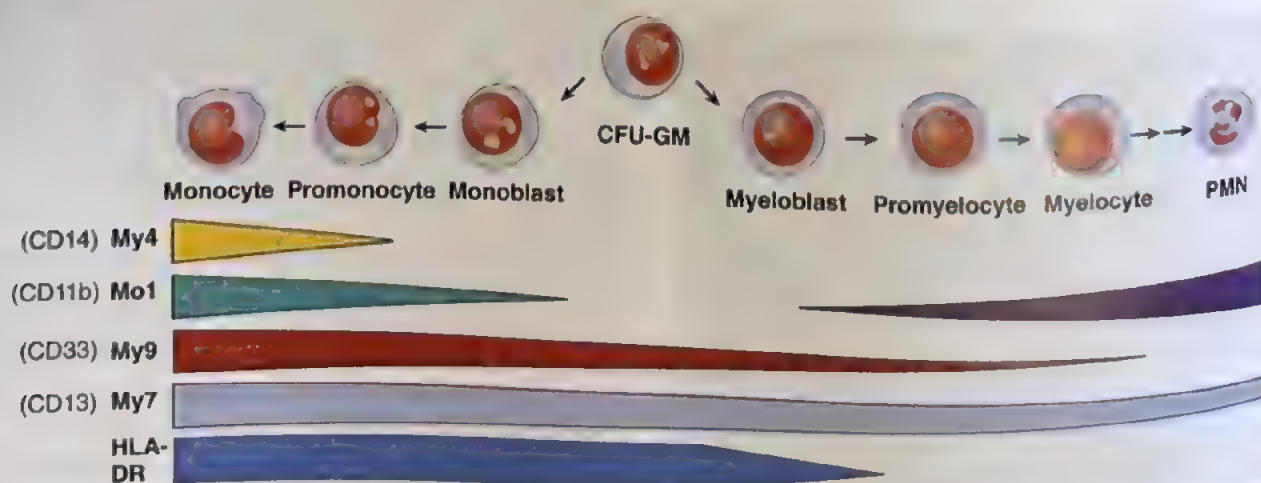


FIGURE 17-8 Distribution of myeloid and monocytic surface antigens with bars depicting the strength of antigen reactivity. The wider the bar, the stronger the reaction, the narrowing indicating a weaker reaction. PMN = polymorphonuclear neutrophil; CFU-GM = colony-forming unit granulocyte macrophage/monocyte.

CD15, CD33, and CD64. B-cell antigens include CD19, CD20, and CD22. T-cell antigens include CD2, CD3, CD4, CD5, CD7, and CD8. Although the immunophenotypes demonstrated by flow cytometry analysis do not correlate with the specific WHO classification of AML with genetic abnormalities, there are certain patterns that can be observed. For example, cases of AML with monocytic differentiation may express monocyte-related surface markers such as CD14, CD4, and CD11c. Promyelocytic leukemia characteristically expresses CD13 and CD33, while being negative for HLA-DR and CD34, a pattern that should warrant evaluation for $t(15;17)$ in a blast population. Acute myeloid leukemia with $t(8;21)$ can express aberrant B-cell marker CD19 in addition to myeloid markers.^{13,14}

Cell marker studies can also be directed at cytoplasmic antigens using either immunohistochemistry or flow cytometry. When using flow cytometry, an additional step must be taken to fix and permeabilize the cells to allow antibodies to enter through the cell membrane and into the cytoplasmic space. Often, the quantity of antigen on the cell surface and in the cytoplasm varies, and some antigens are exclusively cytoplasmic. Plasma cell neoplasms, for example, often have very weak surface and strong cytoplasmic immunoglobulin-staining intensity.

Cytoplasmic markers are very useful in assessing cell lineage. In T lymphoblastic leukemia, cytoplasmic CD3 is present early in T-cell development, and strong expression is seen in most cases of precursor T-cell lymphoblastic leukemia. Likewise, the presence of cytoplasmic CD22 and CD79a aids in defining B-cell lineage in B lymphoblastic leukemia. Cytoplasmic myeloperoxidase is a myeloid marker often used as part of a flow cytometric panel, in addition to the traditional myeloid markers CD13 and CD33, to help establish myeloid differentiation.⁸

Antibodies can also be directed at nuclear antigens using the same cell membrane permeabilization procedure. Terminal deoxynucleotidyl transferase (TdT) is a unique nuclear enzyme (DNA polymerase) present in stem cells and precursor B- and T-lymphoid cells.¹⁵ High levels are found in the majority (90%) of the lymphoblastic leukemias, including both B- and T-lineage leukemias. However, TdT is not lineage-specific and can be seen in at least 18% of AML cases.¹⁵ It is more common in acute myeloid leukemia with minimal differentiation, where it can be seen in approximately 50% of cases.¹³ The number of myeloblasts that express TdT, however, is usually significantly lower than in lymphoblasts.¹⁵ Examples of an acute myeloid leukemia and B lymphoblastic leukemia flow cytometry scatter plots are seen in Figures 17-9 and 17-10.

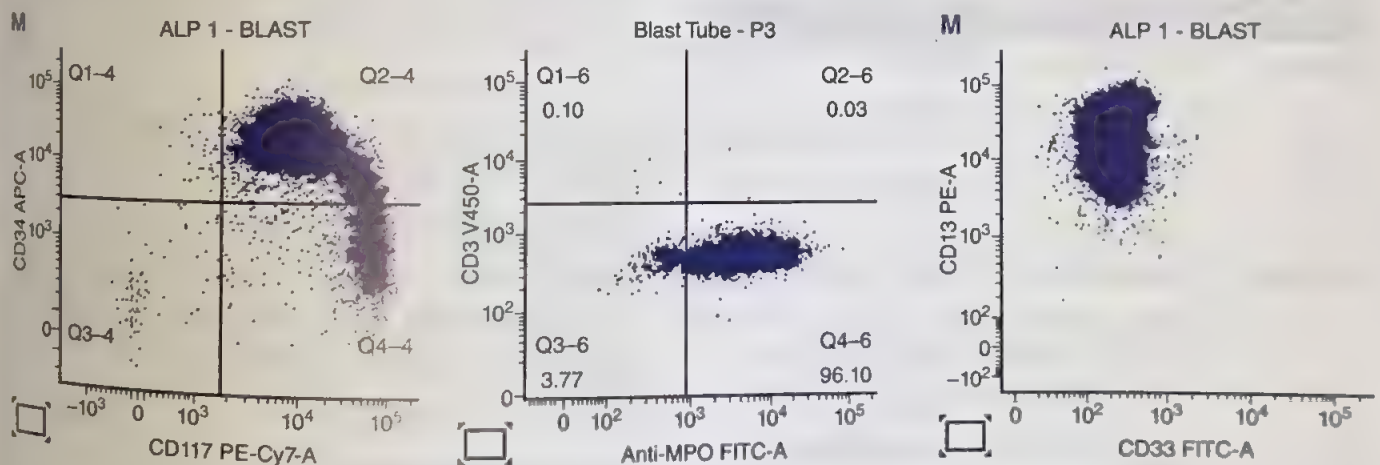


FIGURE 17-9 Acute myeloid leukemia flow example.

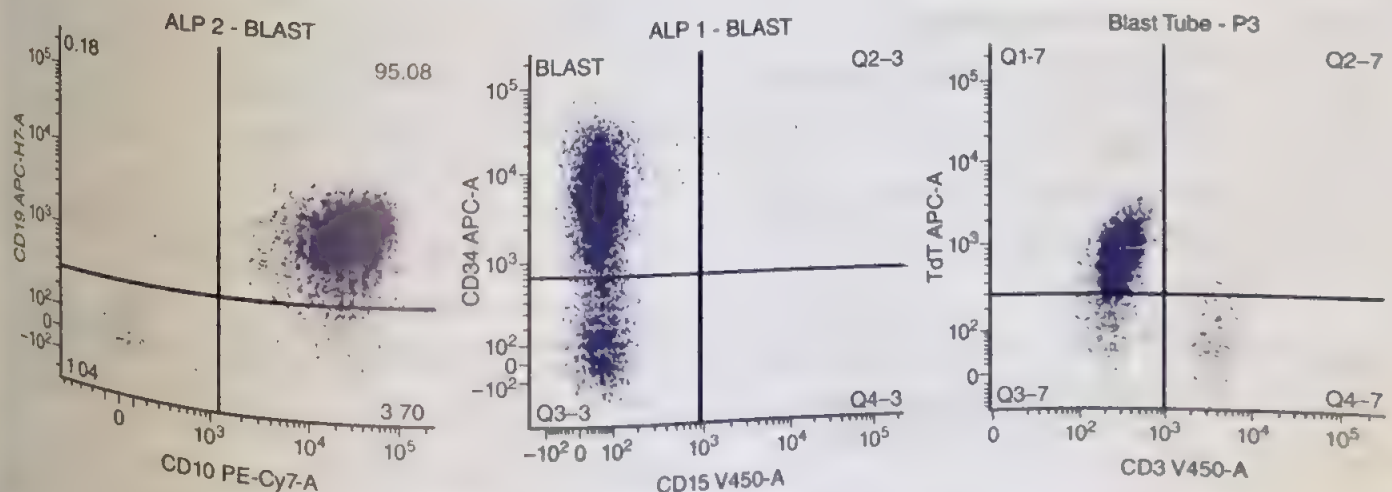


FIGURE 17-10 B Lymphoblastic leukemia flow example.

Genetic Analysis

The genetic analysis of leukemic cells is an essential component in the evaluation of the newly diagnosed leukemic patient. It plays a major role in diagnosis, subclassification, prognosis, selection of appropriate therapy, and monitoring the effects of therapy. In the WHO classification, several chromosomal abnormalities have been associated with distinct forms of leukemia. Classic examples of this are the Philadelphia chromosome t(9;22) associated with CML and the translocation t(15;17) consistently observed in acute promyelocytic leukemia. The WHO classification for AML includes entities with recurrent cytogenetic abnormalities, each with clinical significance and important prognostic information, which will be discussed later in this chapter. Genetic analysis may be carried out in different ways, including cytogenetic karyotyping, florescence in situ hybridization (FISH), polymerase chain reaction (PCR), and/or next generation sequencing analysis. Typically, a combination of testing methodologies are currently used for classification, prognosis/predictive value, and to evaluate for treatment options. For a summary of genetic risk stratification in AML and B-ALL see Tables 17–9 and 17–10.

TABLE 17-9 Genetic Risk Stratification for AML	
Favorable	t(8;21)(q22;q22.1); RUNX1-RNXT1 inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11 Mutated NPM1 without FLT-ITD or with FLT-ITD ^{low} Biallelic mutated CEBPA
Intermediate	Mutated NPM1 and FLT3-ITD ^{HIGH} Wild Type NPM1 without FLT3-ITD or with FLT3-ITD ^{low} (without adverse-risk genetic lesions) t(9;11)(p21.3;q23.3); KMT2A- MLLT3 Other abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); DEK-NUP214 t(v;11q23.3); KMT2A rearranged inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2): GATA2, MECOM(EVL1) -5 or del5q; -7; -17/abn(17p) Complex Karyotype (≥3), monosomy karyotype Wild type NPM1 and FLT3-ITD ^{high} Mutated RUNX1 Mutated ASXL Mutated TP53

Adapted from NCCN Guideline version 1.2022 Acute Myeloid Leukemia (Age >18 years)²¹

TABLE 17-10 Genetic Risk Factor for B-ALL	
Good Risk	Hyperdiploidy (51–65 chromosomes) <ul style="list-style-type: none">• Cases with trisomy of chromosomes 4, 10, and 17 appear to have the most favorable outcome• t(12;21)(p13;q22.1): ETV6-RUNX1
Poor Risk	Hypodiploidy (<44 chromosomes) KMT2A rearrangement (t[4;11] or others) t(v;14q32/IgH) t(9;22)(q34.1;q11.2) Complex karyotype (5 or more chromosomal abnormalities) BCR-ABL1 like (Ph-like) ALL <ul style="list-style-type: none">• JAK-STAT (CRLF2r, EPORr, JAK1/2/3r, TYK2r, mutations of SH2B3, IL7R, JAK1/2/3• ABL class (rearrangements of ABL1, ABL2, PDGFRA, PDGFRB, FGFR)• Other (NTRKr, FLT3r, LYNr, PTK2Br) Intrachromosomal amplification of chromosome 21 (IAMP21) t(17;19): TCF3-HLF fusion Alterations of IKZF1

Adapted from NCCN Guidelines Version 4.2001 Acute Lymphoblastic Leukemia²¹

▶ ADVANCED CONTENT

It should be noted that a diagnosis of acute myeloid leukemia can be made with <20% blasts in cases with t(8;21), inv(16), t(16;16), and promyelocytic leukemia/retinoic acid receptor alpha (*PML-RARA*) fusion¹³ (an abnormal fusion gene), since all of these are considered disease defining.

Cytogenetics and FISH
Karyotyping evaluates stained chromosome metaphase preparations to detect the numeric and structural karyotype (Greek *karyon*, meaning nucleus, and *typos*, meaning mark) abnormalities. Normal human cells have 46 chromosomes and are diploid, having two alleles of each gene. That is, they have two haploid sets of 23 chromosomes.
Karyotyping can detect numeric and structural rearrangements, including translocations, inversions, deletions, duplications, and isochromosomes. Translocations result from a reciprocal interchange of portions of two nonhomologous chromosomes and are the most common structural chromosomal abnormalities. A number of these are associated with distinct subgroups of AML or ALL and have prognostic significance. Table 17–11 lists recurrent genetic abnormalities associated with acute leukemia.

TABLE 17-11 Recurrent Genetic Abnormalities Associated With Acute Leukemia²¹

Chromosome Abnormality	Characteristic Findings	Involved Genes on Respective Chromosomes
AML with t(8;21)	Large blasts, perinuclear clearing (hofs), Auer rods (characteristically single, long and tapered), may have large pseudo-Chediak-Higashi granules May frequently express CD19 and PAX-5 Associated with a favorable prognosis and good response to therapy	<i>RUNX1-RUNX1T1</i>
AML with inv(16) or t(16;16)	Abnormal eosinophils with purple-violet granules, Auer rods The rearrangement can be cryptic Associated with favorable prognosis	<i>CBFB-MYH11</i>
AML with t(15;17)	Bundles of Auer rods or cytoplasm densely packed with granules Hypogranular variant has bilobed nuclei Characteristically negative for CD34 and HLA-DR Associated with a favorable prognosis	<i>PML-RARα</i>
AML with t(9;11)	Monocytic features >120 variant <i>KMT2A</i> translations Associated with poor prognosis	<i>KMT2A-MLLT3</i>
AML with t(6;9)	Associated with marrow basophilia and multilineage dysplasia Associated with poor prognosis	<i>DEK-NUP214</i>
AML with inv(3) or t(3;3)	Associated with dysplastic megakaryocytes and multilineage dysplasia Associated with poor prognosis	<i>GATA2, MECOM</i>
AML with t(1;22)	Infants without Down Syndrome, megakaryoblasts with blebs/pseudopods May express CD41, CD61, and CD42b High-risk disease	<i>RBM15-MKL1</i>
AML with t(9;22)	Provisional diagnosis of de novo AML with no prior evidence of CML Can have aberrant expression of CD19, CD7, TdT Associated with poor prognosis	<i>BCR-ABL1</i>
B-ALL with t(9;22)	More common in adults than children CD25 frequently expressed in adults Frequent expression of CD13 and CD33 Poor prognosis but TK inhibitors have improved outcome	<i>BCR-ABL1</i>
B-ALL with t(v;11q23.3)	Most common B-ALL in infants <1 year Associated with poor prognosis	<i>KMT2A-rearranged</i>
B-ALL with t(1;19)	Precursor B-cell ALL Associated with poor prognosis	<i>TCF3-PBX1</i>
B-ALL with t(12;21)	Can be cryptic by karyotype CD13 frequently expressed Associated with favorable prognosis	<i>ETV6-RUNX1</i>

t = translocation; inv = inversion
Credit: Kennedy VE, Smith CC. FLT3 Mutations in Acute Myeloid Leukemia: Key Concepts and Emerging Controversies. Front Oncol. 2020 Dec 23;10:612880.

Current standard karyotyping, however, is time consuming and will fail to detect chromosomal abnormalities in 40% to 50% of AMLs, as some abnormalities can be cryptic (meaning undetectable by cytogenetic analysis).¹⁶ Thus, other methods such as FISH are routinely done today. FISH has several advantages: a faster turnaround time, the ability to use cells in either metaphase or interphase, the ability to use paraffin-embedded tissue, and the ability to detect some abnormalities not seen by conventional karyotyping.

ADVANCED CONTENT

For example, acute myeloid leukemia with *inv*(16) and B lymphoblastic leukemia with *t*(12;21) can be cryptic by conventional cytogenetics; therefore, an evaluation by FISH is recommended. FISH analysis in acute leukemia typically includes a panel of probes that can help identify translocations such as *BCR/ABL t*(9;22), specific gene rearrangements such as *MLL* rearrangements, and gene amplifications (for example, *RUNX1* amplifications). Their clinical significance and important prognostic information will be discussed later in this chapter. Additionally, molecular studies can detect specific gene mutations such as *NPM1* and *FLT3* mutations that are not detectable by conventional cytogenetic karyotyping or FISH studies.

Molecular Studies

The molecular genetic basis for acute leukemia continues to evolve, and specific gene mutations lead to specific classifications, which can have prognostic significance, help detect minimal residual disease, or have target therapy implications.

ADVANCED CONTENT

Gene mutations associated with specific AML classifications include AML with mutated *NPM1*, AML with biallelic mutation of *CEBPA*, and AML with mutated *RUNX1*. *NPM1* mutations, which can be seen in ~30% of AML cases, are associated with a good prognosis in the absence of *FLT3* mutations. Other typically tested genes in AML include *TP53* and *ASXL1* mutations, which have been associated with poor prognosis.^{17,18} In core-binding factor AMLs such as AML *t*(8;12), the presence of a *KIT* mutation is associated with a poor prognosis.¹⁹ The enzyme isocitrate dehydrogenase (*IDH1*) is mutated in a variety of cancers, including AML. *IDH1* mutations can be seen in 6% to 16% of de novo AML cases and *IDH2* in 8% to 19% of de novo AML cases.²⁰ Although their prognostic significance remains controversial, targeted *IDH* inhibitor therapies are available for these mutations.

Mutations in the tyrosine kinase *FLT3-ITD* gene have both prognostic and treatment implications in acute myeloid leukemia. *FLT3-ITD* mutations are common in acute

myeloid leukemia and can be present in 30% of AML cases.²¹ Mutations lead to constitutive activation of the *FLT3* receptor and are associated with a more aggressive behavior and higher relapse rates after traditional chemotherapy.²² However, the prognostic effect of *FLT3-ITD* mutations is related to the allele ratio defined by the ratio of *ITD*-mutated alleles to the wild-type allele and insertion site. Both an AR ≥ 0.5 (*FLT3-ITD*^{high}) and insertion site in *TKD1* are associated with an unfavorable prognosis. However, *FLT3-ITD* mutations with an AR < 0.5 (*FLT3-ITD*^{low}) and concurrent *NPM1* mutation do not confer an unfavorable prognosis in the absence of other high-risk mutations.²¹

The presence of *FLT3* mutations can be gained or lost during disease relapse or progression.²³ For this reason, it may not be helpful to evaluate for minimal residual disease, and evaluation for *FLT3* mutations may have to be reassessed over time.

The prognostic significance of *FLT3-TKD* mutation remains controversial, and its presence does not alter risk assessment currently. However, both mutations are typically tested due to the development of *FLT3* inhibitors. First generation inhibitors not only target *FLT3* but also target a variety of other kinases. This allows a first generation inhibitor to inhibit both *FLT3-TKD*- and *FLT3-ITD*-mutated receptors. In contrast, second generation inhibitors are more potent and specific, and are only active against the *FLT3-ITD* mutations.²¹

It should be noted that a diagnosis of acute myeloid leukemia can be made with $< 20\%$ blasts in cases with *t*(8;21), *inv*(16), *t*(16;16), and promyelocytic leukemia/retinoic acid receptor alpha (*PML-RARA*) fusion¹³ (an abnormal fusion gene), since all of these are considered disease defining.

Next generation sequencing is now available and allows testing for a broad spectrum of gene mutations including the mutations discussed previously for AML. Panels with a variety of mutations associated with acute lymphoblastic leukemia, which are discussed later in this chapter, are also available. Overall, molecular studies can help identify mutations that are not detected by conventional cytogenetics or FISH studies and can be useful for evaluation of minimal residual disease. Examples include PCR-based assays to detect fusion genes such as *BCR/ABL1*, *PML/RARA*, and NGS-base assays to detect clonal rearrangements in immunoglobulin heavy chain genes in B-ALL and T-cell receptor genes in T-ALL.

Six Major Categories of the WHO Classification

AML With Recurrent Genetic Abnormalities

Recurrent genetic abnormalities include balanced chromosomal rearrangements and inversions involving transcription factors that are associated with distinct morphological, immunophenotypic, and clinical features. It should be noted that the WHO maintains that AML with *t*(8;21)(q22;q22.1) and AML with *inv*(16)(p13.1;q22) or *t*(16;16)(p13.1;q22), and

acute promyelocytic leukemia with $t(15;17)(q22;q11-12)$ are considered to be acute leukemia regardless of the blast percentage. In the revised fourth edition of the WHO classification published in 2017, AML with gene mutations is also included as a category based on the presence of specific mutations detected by molecular studies.

AML With $t(8;21)(q22;q22.1);(RUNX1-RUNX1T1)$

This translocation occurs in up to 1% to 5% of cases of AML. The *RUNX1-RUNX1T1* (also called *ETO-AML1*) translocation occurs chiefly in younger patients and often in cases of AML with maturation (FAB M2). The translocation involves the fusion of the *RUNX1* (also called *AML1*) gene on chromosome 21 that encodes the alpha subunit of the core-binding factor with the *RUNX1T1* (also called *ETO*) gene on chromosome 8. This results in the creation of *RUNX1-RUNX1T1* fusion transcript that regulates transcription and blocks differentiation to promote leukemic progression.²⁴ FISH and RT-PCR assays for this abnormality are quite reliable.¹³ The characteristic morphological finding in this entity is a single long Auer rod with tapered ends that may be seen within blasts and mature neutrophils. Myeloblasts often containing abundant basophilic cytoplasm with azurophilic granules that may be very large (pseudo-Chediak-Higashi granules). Eosinophilic precursors are often increased with normal morphological features. Variable dysplastic features may be seen among neutrophils and precursors, but dysplasia is uncommon in other cell lines. Expression of myeloid markers, such as CD13, CD34, and MPO, are typical, but in addition, these leukemias express markers of the lymphoid lineage, most frequently CD19 and *PAX5*. Additional chromosomal abnormalities are common including loss of a sex chromosome and 9q deletion. The $t(8;21)$ is generally associated with a favorable prognosis and good response to therapy containing high-dose cytosine arabinoside.¹³ Adverse prognostic factors include the presence of KIT mutation in adults and CD56 expression.

AML With $inv(16)(p13.1;q22)$ OR $t(16;16)(p13.1;q22);(CBFB/MYH11)$

Pericentric inversion of chromosome 16 and $t(16;16)(p13.1;q22)$ is associated with another type of AML that has a favorable prognosis, also called acute myelomonocytic leukemia with abnormal eosinophilia and M4eo under the FAB system. The $inv(16)$ results in the fusion of the core binding factor beta (*CBFB*) gene on 16q22 with the *MYH11* gene on 16p13 that codes for a smooth muscle myosin heavy chain.¹³ This leads to the sequestration of much of the *CBFB* protein in the cytoplasm, preventing its function as a transcription factor. This type of leukemia characteristically shows an abnormal eosinophil component as well as granulocytic and monocytic differentiation. The abnormal eosinophils appear immature and show large, purple-violet granules. Similar to patients with AML $t(8;21)$, AML $inv(16)$ patients achieve higher rates of remission with therapy containing high-dose cytosine arabinoside. Inversion 16 is a subtle rearrangement that may be overlooked by conventional cytogenetic analysis; thus, FISH or RT-PCR is recommended to detect this alteration. Advanced age, elevated white blood cell count, *FLT3* mutations, and trisomy 8 are associated with worse outcomes. KIT

mutations in adults are associated with higher risk of relapse and worse survival; however, the prognostic associations are not as significant as in AML with $t(8;21)$.

Acute Promyelocytic Leukemia With *PML-RARA*

Acute promyelocytic leukemia (APL; also known as AML M3 under the FAB system) is an acute myeloid leukemia characterized by abnormal promyelocytes (Fig. 17-11), the *PML-RARA* fusion gene transcript, and high cure rates with treatment. The abnormal promyelocytes are rich in thromboplastic substances that, if released, trigger disseminated intravascular coagulation (DIC). APL may present with hemorrhagic manifestations, including petechiae, small ecchymoses, hematuria, bleeding from venipuncture and bone marrow sites, CNS, and pulmonary hemorrhages.²⁵ The most consistent coagulation abnormalities include a prolonged prothrombin time and thrombin time, elevated fibrin degradation products, and decreased amounts of plasma fibrinogen. Thrombocytopenia is almost universally present. Schistocytes are sometimes evident on the peripheral blood smear. Left untreated, coagulopathy is associated with significant early death rates.

Screening for APL by morphological analysis requires a high index of suspicion and is of paramount importance for rapid diagnosis of this time-sensitive disease. The characteristic morphological finding in both the hypergranular and microgranular variants is a reniform (kidney-shaped) or bilobed nucleus. This finding is seen in at least a subset of leukemic cells in hypergranular APL, which often has greatly variable nuclear size and shape. The abnormal promyelocytes in this variant show heavy granulation, sometimes obscuring the nucleus, and abundant cytoplasm. Auer rods are frequently seen, and some cells may contain bundles or stacks of Auer rods. Microgranular APL can be mistaken for acute myelomonocytic or monocytic leukemia since the leukemic cells appear monocytoid with prominent nuclear folding and abundant cytoplasm (Figure 17-12). However, the nucleus of most cells in the peripheral blood is reniform or bilobed. Granulation of these cells is scant or absent, although occasional cells with heavy granulation are almost always present. The leukocyte count is often markedly elevated in the

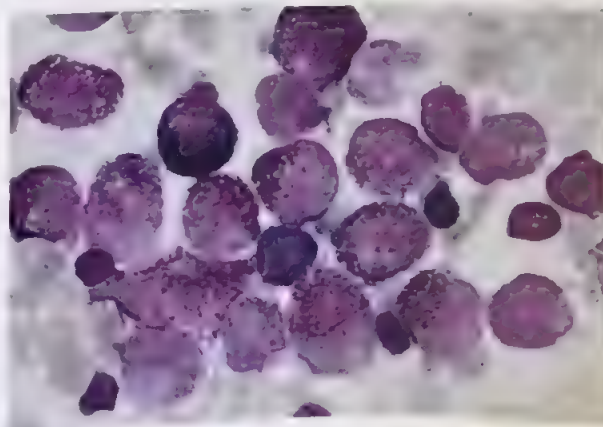


FIGURE 17-11 Acute promyelocytic leukemia (APL), M3, bone marrow



FIGURE 17-12 Acute "microgranular" promyelocytic leukemia, M3, peripheral blood.

microgranular variant of APL. The bone marrow aspirate may reveal a morphological pattern that more closely resembles typical APL.

Flow cytometry is a very useful adjunct to identifying APL. The characteristic immunophenotype of the promyelocytes/blasts shows low or absent expression of CD34 and HLA-DR with bright, homogenous expression of CD33 and MPO and heterogenous expression of CD13. CD56 expression in APL has been associated with a worse outcome.²⁶ By cytochemistry, MPO and Sudan black (Fig 17-13) are also strongly positive in leukemic cells, including the microgranular variant.

In addition to the characteristic morphology, APL contains a translocation that results in the fusion of a transcription factor called *PML* on chromosome 15 with the alpha (α)-retinoic acid receptor gene (*RARA*) on chromosome 17, giving rise to one of the most striking instances of genotype-phenotype correlation in pathology: the hybrid gene, *PML-RARA*.¹³ When morphological and/or immunophenotypic features of APL are identified, the diagnosis of this entity requires confirmation by FISH or RT-PCR analysis to detect the presence of this rearrangement/fusion transcript. Because *RARA* acts as a transcriptional activator, when translocated, it acts as a repressor of transcription leading to a maturation arrest at the

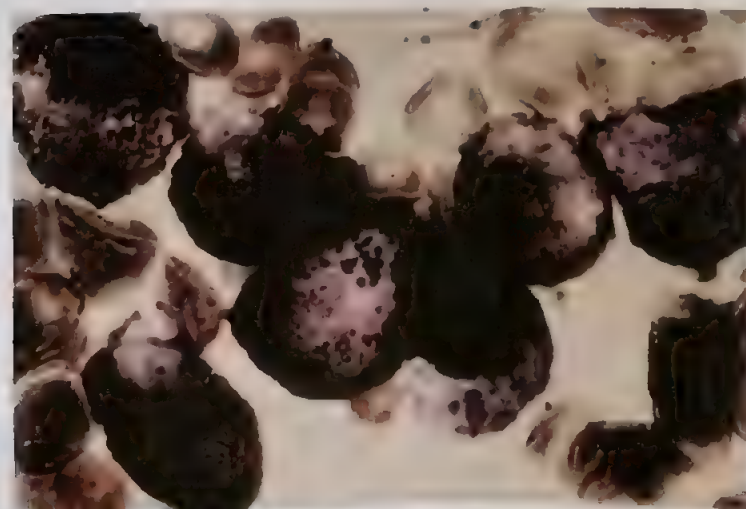


FIGURE 17-13 APL, M3 (Sudan black B stain).

promyelocyte stage.¹³ In the normal state, *RARA* interacts with transcriptional corepressors, and the levels of retinoic acid present are sufficient for overcoming this repression. However, the fusion of *RARA* to *PML* leads to the formation of a corepressor complex that is enhanced.¹³ The formation of this enhanced complex is involved in the oncogenesis of APL through mechanisms that are not entirely understood, but high doses of all-transretinoic acid (ATRA) are effective as a differentiating agent in APL.¹³ ATRA in combination with arsenic trioxide therapy has become the standard therapy for most patients with anthracycline added for high-risk patients. The prognosis for APL treated optimally is more favorable than that for any other AML subtype.

AML With t(9;11)(p21.3;q23.3); *KMT2A-MLLT3* Abnormalities

The *KMT2A* gene (previously called *MLL*) is involved in at least 120 different translocations, many of which are seen in AML, ALL, and MDS.¹³ This diagnostic category should be reserved for cases of *de novo* AML with 11q23.3 balanced translocations involving *KMT2A*. Cases of AML with *KMT2A* translocation and either prior therapy or concurrent myelodysplasia should be categorized as therapy-related myeloid neoplasm and AML with myelodysplasia-related changes, respectively (see later in this chapter).

This subtype is more common in children but can occur at any age. Clinically, patients may present with extramedullary monocytic sarcoma or DIC.¹³ AML with t(9;11) is associated with monocytic differentiation, including monoblasts and promonocytes that can be highlighted by nonspecific esterase. In children, the immunophenotype shows strong expression of CD33, CD4, HLA-DR, and CD65 with lower expression of CD13, CD14, and CD34. Adult cases can express any marker of monocytic differentiation and variable expression of immature markers.

Since some *KMT2A* translocations are subtle by conventional karyotype analysis, FISH or molecular studies may be necessary to identify these variant translocations. Typically, *KMT2A* gene abnormalities denote a poor outcome.²⁷

AML With t(6;9)(p23;q34.1); *DEK-NUP214*

This AML arises from the rearrangement of *DEK* on chromosome 6 with *NUP214* on chromosome 9 resulting in an aberrant transcription factor. The characteristic morphological features of this subtype are bone marrow and peripheral basophilia and multilineage dysplasia including ring sideroblasts in some cases. Immunophenotypically, up to half of cases express TdT and otherwise show a nonspecific myeloid immunophenotype. This AML subtype typically shows a poor outcome.¹³ Most cases show a FLT3-ITD mutation that may be targeted with FLT3 inhibitors.

AML With inv(3)(q21.3;q26.2) OR t(3;3)(q21.3;q26.2); *GATA2-MECOM*

The inversion or rearrangement of the long arm of chromosome 3 results in shifting the *GATA2* enhancer that activates the oncogene *MECOM* at 3q26.2. This results in an acute leukemia with myeloblasts that may show myelomonocytic or megakaryocytic morphology and immunophenotype.

Dysplastic morphological features may be seen in the peripheral blood and bone marrow. Secondary karyotype abnormalities are common and include monosomy 7, 5q deletions, and complex karyotype. Gene mutations that may be seen include *NRAS*, *FLT3*, *KRAS*, *KIT*, *RUNX1*, and *GATA2*.¹³ Clinically, this is an aggressive disease with poor prognosis. In addition, hepatosplenomegaly may be present, while lymphadenopathy is uncommon.

AML (Megakaryoblastic) With t(1;22)

(p13.3;q13.1);*RBM15-MKL1*

This is an uncommon entity and occurs in less than 1% of all AML cases, essentially restricted to infants and young children under 3 years of age without Down Syndrome. Physical exam findings usually reveal marked hepatosplenomegaly, while laboratory studies usually show anemia, thrombocytopenia, and leukocytosis. Like the name implies, this AML shows a megakaryocytic phenotype. By morphology, megakaryoblasts show enlarged, irregular, or indented nuclei with immature chromatin and basophilic cytoplasm with pseudopod or cytoplasmic bleb formation. The bone marrow may also show micromegakaryocytes (dysplastic megakaryocytes) and stromal fibrosis. Immunophenotypically, megakaryoblasts express at least one megakaryocyte marker to include surface and/or cytoplasmic CD41, CD61, and CD42b. In addition, megakaryoblasts may express myeloid markers CD13 and CD33 but are negative for MPO and lymphoid markers.¹³ Clinically, this is an aggressive disease with a worse prognosis compared with pediatric acute megakaryoblastic leukemia without t(1;22).

AML With *BCR-ABL1* (Provisional Entity)

This is a provisional entity in the most recent edition of the WHO Classification published in 2017 and recognizes *de novo* AML without evidence of previous or concurrent chronic myeloid leukemia (CML). Most cases show the p210 fusion, while p190 transcripts are infrequent. Additional cytogenetic abnormalities are common, including monosomy 7, trisomy 8, and complex karyotypes. Unlike typical AML arising from preexisting CML, this acute leukemia shows no evidence of organomegaly or peripheral basophilia. The morphology is nonspecific, while the immunotype is distinct because of the frequent coexpression of CD19, CD7, and TdT with myeloid markers.¹³ Clinically, this is an aggressive subtype of AML, although improved survival may be achieved with tyrosine kinase inhibitors and allogeneic therapies.

AML With Mutated *NPM1*

AML with this mutation often involves exon 12 of *NPM1*. This genetic aberration may be detected by molecular studies or cytoplasmic expression of *NPM1* protein as a surrogate marker. This subtype of AML is usually associated with a normal karyotype; however, a small subset of cases shows trisomy 8 and 9q deletions. Morphologically, monocytic differentiation is common and morphological dysplasia may be seen. The immunophenotype may show immature myelocytes or monocytic differentiation. This AML typically shows a good response to induction chemotherapy. Cases with a normal karyotype and no *FLT3-ITD* mutations show a favorable

prognosis. Poor prognostic markers include the presence of *FLT3-ITD* and *DNMT3A* mutations.¹³

AML With Biallelic Mutation of *CEBPA*

AML with biallelic *CEBPA* mutations should be evaluated for a germline mutation and predisposition to develop AML. These cases often show a normal karyotype, although 9q deletion may be present without adversely affecting the prognosis. *GATA2* and *FLT3-ITD* mutations may also be seen with unclear prognostic significance.¹³ No distinctive morphological or immunophenotypic features are seen in this subtype of AML; however, most cases show features of AML with or without maturation as well as multilineage dysplasia. This subtype of AML is associated with a favorable prognosis.

AML With Mutated *RUNX1* (Provisional Entity)

This subtype is reserved for *de novo* cases of AML that do not meet criteria for AML with recurrent genetic abnormalities, therapy-related myeloid neoplasms, or AML with MDS-related changes. No specific morphological or immunophenotypic features have been reported. This subtype of AML is associated with poor overall survival in some studies. Cases with *RUNX1* and *ASXL1* mutations are associated with a worse prognosis.¹³

AML With Myelodysplasia-Related Changes

AML with myelodysplasia (MDS)-related changes is an acute leukemia with greater than 20% blasts and evidence of dysplasia. Morphologically, dysplasia must be present in greater than 50% of the cells in at least two lines, and it must be in a pretreatment specimen.¹³ In addition, AML arising in a preexisting MDS or MDS/MPN as well as the presence of an MDS-related cytogenetic abnormality qualifies for this diagnosis. Most often, patients present clinically with severe pancytopenia. This is a morphologically heterogeneous disease, and these features are reflected in the immunophenotype, which is highly variable, often showing aberrant antigen expression. Cytogenetic alterations are often complex and often include the loss of chromosomes 5q and 7q. The prognosis for this subtype of AML is not favorable. The presence of a *TP53* mutation suggest a worse prognosis.¹³

Therapy-Related Myeloid Neoplasms

This category encompasses acute myeloid leukemia, myelodysplastic syndrome, and myelodysplastic/myeloproliferative neoplasms that arise in the setting of prior cytotoxic chemotherapy (including alkylating agents and topoisomerase II inhibitors) and radiation therapy. Two major clinical subtypes of therapy-related AMLs are recognized: (1) alkylating agent/radiation-related, and (2) topoisomerase II inhibitor-related.¹³ The development of leukemia is more rapid after topoisomerase II inhibitor therapy, with a reported mean of about 33 to 34 months after beginning chemotherapy, compared with about 5 to 6 years after alkylating agent/radiation.¹³

Therapy-related AML and MDS related to alkylating agents/radiation therapy typically show clonal evolution from MDS, frequently harboring deletions or translocations involving 5q and 7q, in addition to other chromosomal abnormalities and mutated *TP53*.¹³ These leukemias are generally associated with a very poor outcome and short survival time.

Morphologically, multilineage dysplasia is typically seen in the peripheral blood and bone marrow. The peripheral blood often shows basophilia, and the bone marrow often contains increased reticulin fibrosis. Immunophenotypically, the myeloid blast population shows no specific pattern, but it may show aberrant expression of CD7 and CD56.

In contrast, AML arising from topoisomerase inhibitor treatment have different cytogenetic abnormalities, the most common of which is a translocation involving 11q23 (*KMT2A*, previously called *MLL*).¹³ Other reported genetic abnormalities in AML after topoisomerase treatment include t(8;21), inv(16), and even APLs with t(15;17).²⁴ Lastly, it may be that these patients have a better outcome than those with alkylating agent/radiation-associated MDS or AML.

Acute Myeloid Leukemia, Not Otherwise Specified

AML With Minimal Differentiation

Also known as FAB M0, cases of acute myeloid leukemia with minimal differentiation show no definitive myeloid differentiation by conventional morphological and cytochemical analysis (Fig. 17-14). These AMLs have primitive leukemic blasts that show no distinctive myeloid morphological features and lack reactivity (less than 3% of blasts staining positive) with the conventional battery of cytochemical stains (myeloperoxidase, Sudan black B, NSE).¹¹ Accordingly, flow cytometry or another form of immunophenotyping is required for the diagnosis. Typically, these minimally differentiated AMLs exhibit immunological reactivity for at least two myeloid lineage-specific antigens (CD33, CD13, CD117) in the absence of lymphoid antigens, particularly cCD3, cCD79a, and cCD22. Rarely, blasts may also express a lymphoid antigen, but the expression is dimmer than that seen in lymphoid malignancies, and there should be more myeloid antigens expressed. Generally, minimally differentiated AML is negative for expression of antigens of myelomonocytic differentiation, such as CD14 and CD15.¹³ By cytogenetic studies, complex karyotypes and unbalanced abnormalities are the most common findings. In the differential diagnosis for minimally differentiated AML, the following is included: ALL,

acute megakaryoblastic leukemia, and acute leukemia of ambiguous lineage. The prognosis appears to be poor compared with other types of AML.¹³

AML Without Maturation

Known as AML M1 under the FAB classification, acute myeloid leukemia without maturation is composed of predominantly poorly differentiated myeloblasts (Fig. 17-15). Importantly, there should be no significant evidence of maturation to more mature myeloid cells such as neutrophils and maturing granulocytic cells comprise less than 10% of the nucleated cells. The nucleus of the myeloblast typically has a fine, lacy chromatin pattern and distinct nucleoli. The quantity of cytoplasm is usually moderate, and azurophilic granules or Auer rods may be seen. The myeloperoxidase or Sudan black reactions must demonstrate at least 3% positivity in the blast population to document myeloid differentiation. Immunophenotypically, at least one myelomonocytic antigen is expressed (CD13, CD33, CD34, and CD117), but lymphoid antigens are generally absent. No specific genetic abnormalities have been reported. The course of this leukemia is generally described as aggressive.

AML With Maturation

Synonymous with the FAB M2, cases of AML with maturation show a marrow infiltrate with greater than 20% blasts and evidence of maturation. By criteria, there should be more than 10% of cells at different stages of granulocyte maturation (Fig. 17-16). At least 3% of the leukemic cells are myeloperoxidase (Fig. 17-17) or Sudan black positive, and this percentage is usually also much higher.¹³ Monocytes comprise less than 20% of the bone marrow; thus, NSE activity must not exceed 20% of marrow cells.⁶

AML with maturation immunophenotypically shows a pattern of granulocytic differentiation by flow cytometry. Myeloblasts express at least one of the myeloid-associated antigens CD13, CD15, CD33, CD11b, and CD65, while the immature markers CD34, CD117, and HLA-DR may only be expressed in a subset of blasts.¹³ Rarely, aberrant expression of CD2, CD7, CD56, CD4, and CD19 may be seen in the most immature blasts. No specific genetic abnormalities have

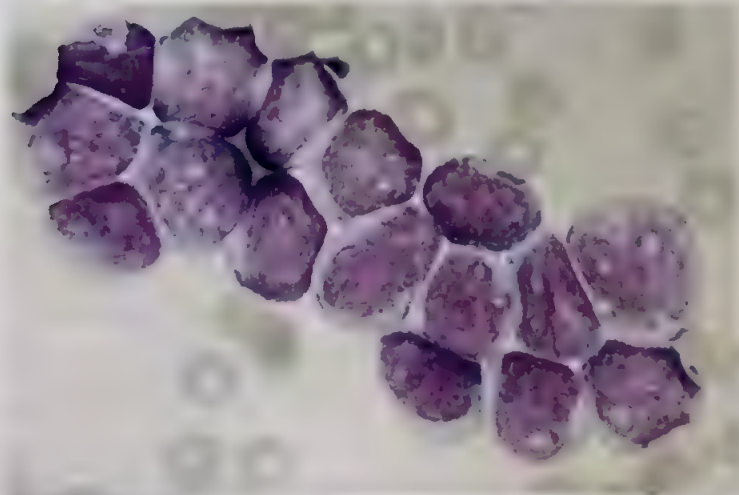


FIGURE 17-14 Acute myeloid leukemia (AML) with minimal differentiation, M0, bone marrow.



FIGURE 17-15 AML without maturation, M1, peripheral blood.

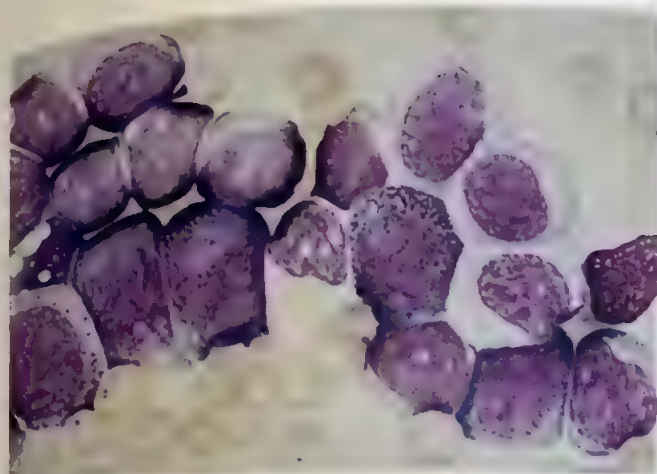


FIGURE 17-16 AML with maturation, M2, bone marrow.

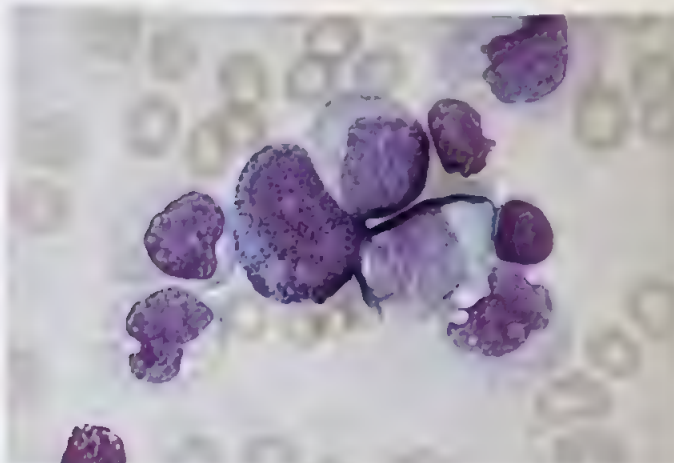


FIGURE 17-18 Acute myelomonocytic leukemia (AMML), M4, bone marrow.

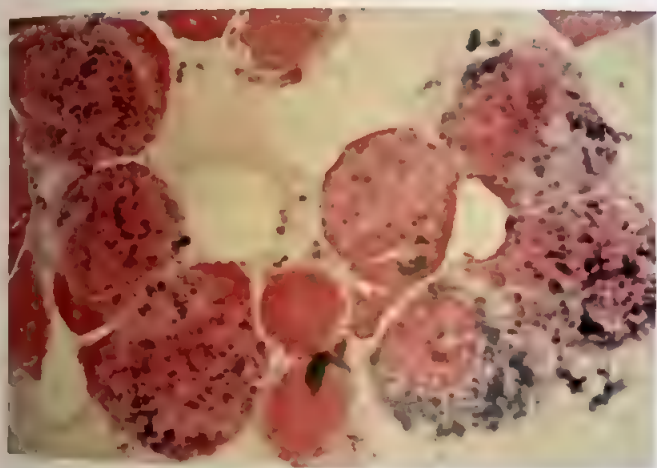


FIGURE 17-17 AML, M2 (myeloperoxidase stain).

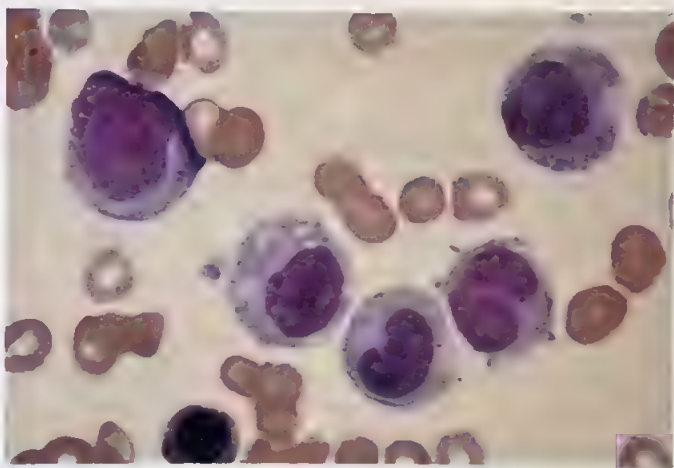


FIGURE 17-19 Promonocytes in AMML, M4, peripheral blood.

been reported in this subtype. Survival rates are variable, and evidence shows that AML with maturation responds to aggressive therapy.

Acute Myelomonocytic Leukemia

Acute myelomonocytic leukemia (AMML), M4 in the FAB system, is characterized by both neutrophilic and monocytic differentiation. By WHO criteria, not only must blasts be greater than or exceed 20%, but neutrophils and precursors, as well as monocytes and precursors, must each comprise equal to or greater than 20% of the bone marrow cellularity.¹³ Morphologically, monoblasts have round nuclei, prominent nucleoli, and delicate chromatin, surrounded by a basophilic cytoplasm that may contain fine azurophilic granules (Fig. 17-18). The distinction between monoblasts and promonocytes is often difficult, but promonocytes generally have a more convoluted nucleus and a lower nuclear:cytoplasmic ratio than do monoblasts (Figure 17-19) as well as a more granular cytoplasm that is less basophilic and more vacuolated than monoblasts. At least 3% of the myeloid blasts must positively stain for myeloperoxidase. Generally, monoblasts, promonocytes, and monocytes will stain positively with NSE.

AMML variably expresses the myeloid antigens CD13, CD33, CD15, and CD65 in a subset as well as a population expressing antigens of monocytic differentiation including CD11b, CD11c, CD14, CD64, and CD4.¹³ A subset of blasts may express the immature markers CD34 and CD117. Nonspecific genetic abnormalities associated with myeloid neoplasms such as trisomy 8 have been reported. AMML may respond to aggressive therapy and has a prognosis similar to that of other AMLs.

Acute Monoblastic and Monocytic Leukemia

Synonymous with FAB M5a and M5b, acute monoblastic and acute monocytic leukemias are leukemias characterized by a preponderance of cells of monocytic lineage. In these cases, cells of monocytic lineage (monoblasts, promonocytes, and monocytes) must comprise greater than 80% of the leukemic cells (not overall marrow cells).¹³ Like the FAB criteria, the WHO further subdivides leukemias of monocytic lineage based on the relative proportion of monoblasts and promonocytes. In cases of acute monoblastic leukemia (FAB M5a, Fig. 17-20), monoblasts comprise the majority of monocytic cells (typically monoblasts are greater than 80% of the monocytic cells in monoblastic leukemia).¹³

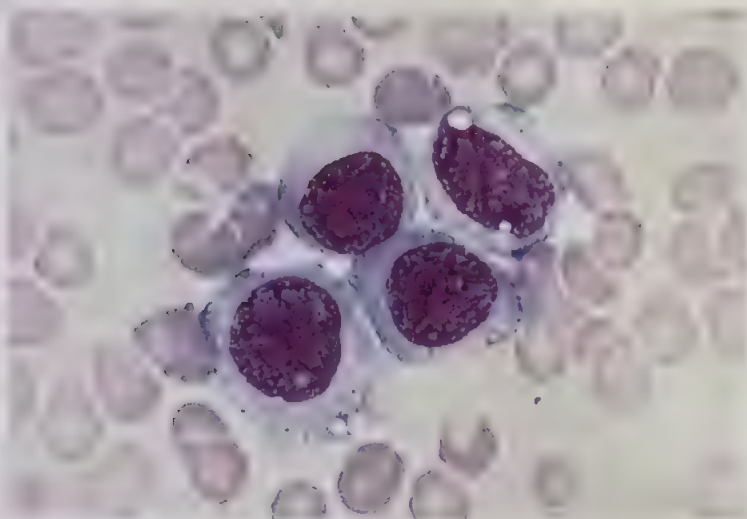


FIGURE 17-20 Acute monocytic leukemia, M5a, peripheral blood.

In contrast, in acute monocytic leukemia (FAB M5b), the majority of monocytic cells are promonocytes or monocytes (Fig. 17-21).

Acute monoblastic and monocytic leukemia both have distinctive clinical manifestations associated with the monocyte's propensity to migrate to extramedullary sites. Skin and gum involvement are particularly characteristic (Fig. 17-22). Lymphadenopathy frequently occurs, and sometimes the spleen and liver are markedly enlarged. CNS involvement also has an increased incidence in these patients.²⁵

The morphological features of the constituent cells have been described earlier in the section on AMML. Typically, monoblasts and promonocytes are strongly positive for NSE.²⁵ Monoblasts are usually not myeloperoxidase positive, whereas promonocytes are weakly to mildly positive. The immunophenotype of these leukemias expresses myeloid antigens (CD13, CD33, and CD117) and expresses at least two markers of monocytic differentiation (CD14, CD64, CD4, and lysozyme).¹³ The immature markers CD117 and HLA-DR are often expressed, while CD34 is expressed in only a minority of cases. While some differential expression of myeloperoxidase may be seen by flow cytometry (i.e., myeloperoxidase antigen may be seen in acute monocytic leukemia rather than

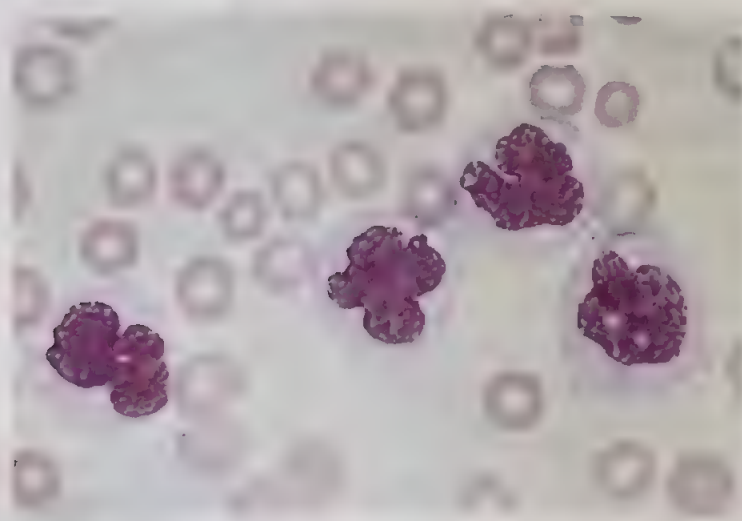


FIGURE 17-21 Acute monocytic leukemia, M5b, peripheral blood.



FIGURE 17-22 Gum hypertrophy: A clinical manifestation of acute leukemia (M5).

acute monoblastic leukemia), these findings alone are not able to discern specific differences between these two subtypes of leukemia.

Nonspecific genetic abnormalities associated with myeloid neoplasms have been reported. Interestingly, the t(8;16) (p11;p13) abnormality is associated with hemophagocytosis by leukemic cells and is also associated with coagulopathy. In general, acute monoblastic and monocytic leukemia follows an aggressive course.

Pure Erythroid Leukemia

Pure erythroid leukemia, which is equivalent to FAB M6, is characterized by an abnormal proliferation of immature erythroid precursors and, generally, an aggressive clinical course.

The neoplastic population is composed of immature erythroid precursors comprising greater than 80% of the cellularity with at least 30% proerythroblasts and no significant myeloblastic component.¹³ The previously recognized erythroleukemia (M6a) was diagnosed based on erythroid precursors comprising 50% or greater of the marrow cells and counting myeloblasts as a percentage of nonerythroid cells. These cases are now classified on the basis of the total bone marrow or peripheral blast count irrespective of the erythroid precursor count. They are now diagnosed as myelodysplastic syndrome with excess blasts if the blasts are less than 20% of the marrow or blood cells and usually AML with MDS-related changes if the blast count is 20% or greater.

Morphologically, erythroblasts show proerythroblast morphology consisting of round nuclei, fine chromatin, one or more nucleoli, and deeply basophilic cytoplasm with occasional cytoplasmic vacuolization. However, blasts may have scant cytoplasm and resemble lymphoblasts. Dysplastic features may be seen, including ring sideroblasts and dysmegakaryopoiesis (Fig. 17-23). By cytochemical analysis, the erythroblasts usually express PAS in a block-like pattern, alpha-naphthyl acetate esterase, and acid phosphatase. They also express E-cadherin and CD71. Depending on how immature the erythroid blasts are, they may express glycophorin and hemoglobin A.¹³ Immature markers CD34 and HLA-DR are negative, while CD117 is often

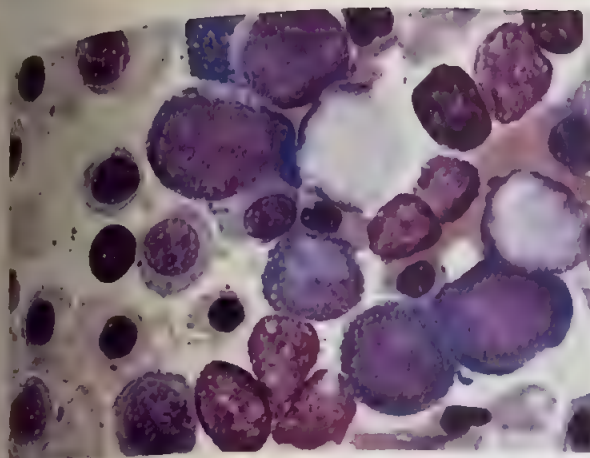


FIGURE 17-23 Pure erythroid leukemia, M6, bone marrow.

positive. They lack myeloperoxidase and Sudan black staining and do not express myeloid antigens. Other antigens such as CD41 and CD61, which are typically megakaryocytic antigens, may be variably expressed. Cytogenetic studies usually reveal a complex karyotype and usually 5q and 7q deletions.¹¹ The median survival for this aggressive subtype is 3 months.

Acute Megakaryoblastic Leukemia

Acute megakaryoblastic leukemia, which is equivalent to FAB M7, is a relatively uncommon form of leukemia characterized by neoplastic proliferation of megakaryoblasts and atypical megakaryocytes (Fig. 17-24). Recognition of this entity was accomplished using platelet peroxidase (PPO) ultrastructural studies. PPO, which is distinct from myeloperoxidase, is specific for the megakaryocytic cell line.¹³ Acute megakaryoblastic leukemia is defined as an acute leukemia with 20% blasts or greater, in which 50% or more of the blasts are of megakaryocytic lineage.¹³ This category excludes cases meeting criteria for AML with recurrent genetic abnormalities and exhibiting megakaryocyte morphology including AML with t(1;22)(p13.3;q13.1), t(3;3)(q21.3;q26.2), or t(3;3)(q21.3;q26.2). In addition,

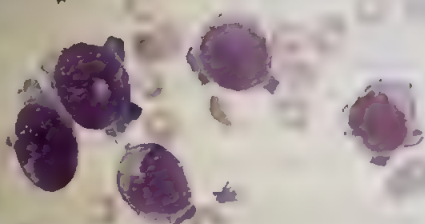


FIGURE 17-24 Acute megakaryoblastic leukemia, M7.

acute megakaryoblastic leukemia occurring in a patient with Down syndrome should be classified as myeloid leukemia associated with Down syndrome.

The blasts observed in acute megakaryoblastic leukemia display a wide range of morphology, from small cells with scant cytoplasm and dense chromatin, to large cells with a moderate amount of cytoplasm and a fine reticulated chromatin pattern. Megakaryoblasts are typically large in size with round or indented nuclei and cytoplasmic projections, or blebs, also known as pseudopods. The peripheral blood may show megakaryoblast fragments, micromegakaryocytes, dysplastic platelets, and dysplastic neutrophils. The bone marrow may show reticulin fibrosis and dysplastic megakaryocytes.¹³

Conventional cytochemistry can suggest the diagnosis of acute megakaryoblastic leukemia but is not definitive. Myeloperoxidase, Sudan black B, and CAE reactions are negative, whereas acid phosphatase and PAS are usually positive.²⁵ Nonspecific esterase shows dotlike positivity.²⁷

The diagnosis of acute megakaryoblastic leukemia is usually made by correlating the morphological findings and demonstrating platelet-specific antigens by immunophenotype. Megakaryoblasts will generally express at least one of the platelet-associated antigens CD41 (glycophorin IIb/IIIa), CD61 (glycophorin IIIa), and CD42b (glycophorin Ib). By flow cytometry, cytoplasmic CD41 and CD61 expression is more specific than surface staining, as platelets may adhere to the surface of blasts that may be misinterpreted as positive staining. CD36 and factor VIII antigen expression are typical. In addition to this, the megakaryoblasts may or may not express the myeloid markers CD13 and CD33, or CD34 and CD45.¹³ It is important to rule out lymphoid differentiation. No unique chromosomal abnormalities are reported in this subtype, and the prognosis is usually worse than other subtypes of AML.

Acute Basophilic Leukemia

In this very rare leukemia, the differentiation is primarily toward the basophilic lineage. Care should be taken to exclude chronic myeloid leukemia (CML) and AML with t(6;9)(p23;q34.1) that may also show increased basophils. The blasts will generally have a moderately basophilic cytoplasm and coarse basophilic cytoplasmic granules similar to those seen in basophils. Numerous immature basophils may also be present, and mature basophils are often few in number. Cytochemically, acute basophilic leukemia shows metachromatic staining with toluidine blue, acid phosphatase, and PAS in some cases but is negative for myeloperoxidase, Sudan black B, non-specific esterase (NSE), and naphthol AS-D chloroacetate esterase (CAE). The lack of CAE by cytochemistry and CD117, tryptase, and CD25 by immunohistochemistry can help differentiate blasts of acute basophilic leukemia from mast cell leukemia. In contrast to normal basophils, blasts may be positive for HLA-DR and negative for CD117. In addition, the blasts express myeloid markers and CD9.¹³ Clinically, features of extramedullary tissue involvement may be seen including skin infiltration, organomegaly, lytic bone lesions, and hyperhistaminemia.

Rare chromosomal abnormalities reported in this subtype include t(3;6)(q21;p21) and abnormalities involving 12p. In addition, t(x;6)(p11.2;q23.3) resulting in *MYB-GATA1* has been reported in male infants with this leukemia.¹³ This is a very rare leukemia; existing information about the outcome of patients suggests a poor prognosis.

Acute Panmyelosis With Myelofibrosis

This, too, is a rare form of acute leukemia representing 1% to 2% of all cases.⁶ It is characterized by an acute panmyeloid proliferation and 20% blasts or more in the setting of bone marrow fibrosis. Clinically, severe pancytopenia is present without splenomegaly, corresponding to the relatively acute onset.¹³ The evolution of this disease process is typically rapid, with patients usually presenting acutely sick with severe constitutional symptoms including fever and bone pain.

The peripheral blood shows pancytopenia with dysgranulopoiesis, abnormal platelets and circulating nucleated red blood cells.¹³ The bone marrow biopsy is hypercellular, with all lineages showing varying amounts of hypercellularity. Bone marrow fibrosis is prominent and typically results in a "dry tap" specimen.¹³ The number of bone marrow blasts is typically between 20% and 25%.

Distinguishing this entity from AML with multilineage dysplasia with fibrosis, acute megakaryoblastic leukemia, and MDS with excess blasts associated with fibrosis may be difficult. Blast percentages and immunophenotype may help, as well as the finding of trilineage hyperplasia in acute panmyelosis with myelofibrosis. In addition, the rapid clinical onset and constitutional symptoms may help distinguish this entity from MDS with excess blasts and myelofibrosis, which progresses relatively slower. Cytogenetic studies, if adequate sample for karyotype analysis is obtained, usually show a complex karyotype. The prognosis for this entity is poor.

Myeloid Sarcoma

Myeloid sarcoma is an extramedullary tumor mass of myeloid blasts with or without maturation that destroys the underlying normal tissue architecture. Myeloid sarcoma is also known as extramedullary myeloid tumor, granulocytic sarcoma, or chloroma. Myeloid sarcoma may occur in many sites in the body, including lymph nodes, skin, gastrointestinal tract, soft tissue, testes, sinuses, brain, and below the periosteum of bones. Myeloid sarcoma is essentially a diagnosis of AML and may occur simultaneously with leukemic AML. Relapsed AML may occasionally present as myeloid sarcoma. Myeloid sarcoma may also occur simultaneously or after treatment with other hematologic and nonhematologic neoplasms. Twenty-five percent of myeloid sarcomas may occur in the absence of leukemic involvement by AML or other myeloid neoplasms. Myeloid sarcoma occurring in the setting of MDS, MDS/MPN, or MPN would be indicative of transformation into an acute blastic phase.¹³

Previous morphological classification of blastic, immature, and differentiated myeloid sarcomas showed no significant clinical or prognostic data, thus classification of myeloid sarcoma relies heavily on immunophenotype.²⁹ Immunophenotypic findings of myeloid sarcoma demonstrate differing

antigen expression based on maturity and myelomonocytic, monoblastic, erythroid, or megakaryocytic differentiation.³ For example, a more immature myeloid sarcoma will show expression of CD13, CD33, CD34, CD68, and CD117, while a promyelocytic differentiation will demonstrate expression of CD34, CD15, MPO, and TdT.¹³ Other examples include expression of CD68 and MPO with lack of CD34 in myelomonocytic subtypes, and monoblastic variants showing expression of CD68 and CD163 with lack of MPO and CD34.¹³ The diagnosis may be difficult in the absence of granulocytic marker expression; thus CD43 and lysozyme may help as they are the most sensitive immunohistochemical markers for myeloid sarcoma.³⁰

A vast majority of cases will show genetic abnormalities that will typically mirror those of the underlying AML or MDS. *NPM1* expression by immunohistochemistry and next generation sequencing typically are associated with MDS/MPN, a monoblastic/myelomonocytic immunophenotype, and frequency of involvement of the skin. Often, genetic abnormalities are more frequently identified in certain body site involvement. For example, inversion 16 can be associated with breast, uterine, or intestinal involvement, while trisomy 8 and *KMT2A-MLL2* fusion is associated more frequently with skin and breast cases.¹³ Prognosis and response to therapy does not appear to be affected by age, sex, histological features, associated myeloid neoplasms, genetic findings, or immunophenotype.³¹ The five-year survival rate for these patients is approximately 50%.³¹

Myeloid Proliferations Related to Down Syndrome

The WHO classification describes two disorders linked with the most common chromosome abnormality among live born infants, trisomy 21, more commonly known as Down syndrome (DS). Due to the genetic abnormality, children with DS have 10- to 100-fold increased risk of developing acute leukemia.¹³ The vast majority (>50%) of acute myeloid leukemia seen in DS children is acute megakaryoblastic leukemia. Development of acute leukemia may occur after a prolonged period of myelodysplastic syndrome-like phase; thus myeloid leukemia associated with DS encompasses both MDS and AML.¹³ These myeloid proliferations associated with DS show unique clinical, morphological, and genetic characteristics that require distinct classification. They include transient abnormal myelopoiesis associated with DS (TAM) and myeloid leukemia associated with DS.

Transient Abnormal Myelopoiesis

Transient abnormal myelopoiesis associated with DS (TAM) is a proliferation of the myeloid lineage resulting in clinical and morphological features identical to acute myeloid leukemia. TAM has been reported in approximately 10% to 13% of newborns with DS.³² Rarely, TAM has been reported in children with trisomy 21 mosaicism; however, it is extraordinarily rare in newborns without chromosome 21 abnormalities. Patients often present at age 3 to 7 days with hepatosplenomegaly, thrombocytopenia, marked leukocytosis, basophilia, and circulating blasts in the peripheral blood. Most infants with this disorder undergo spontaneous remission without treatment within three months.¹³ Approximately 20% to 30%

of these patients will develop acute myeloid leukemia within 1 to 3 years.³⁴

Myeloid Leukemia Associated With Down Syndrome (ML-DS)

Patients with ML-DS typically present within the first 3 to 5 years of life and account for 20% of all pediatric acute leukemias/myelodysplastic syndromes.¹³ Patients may present with refractory cytopenias before manifestation of MDS or acute leukemia.³⁵ As mentioned earlier, acute leukemia may develop in the years following TAM resolution. The leukemic cells typically involve the blood and bone marrow, with extramedullary involvement of the spleen and liver as common findings. Young children with DS and *GATA1* mutations show a favorable prognosis compared with AML in non-DS children; however, a poorer prognosis is associated with older DS children that have a *GATA1* mutation.¹³ Unique chemotherapy and treatment protocols have been established for treatment of ML-DS.

TAM and ML-DS show shared morphological, immunophenotypic, and genetic findings that are discussed later in this chapter. Blasts often have coarse basophilic granules and cytoplasmic blebbing, as seen in megakaryoblasts (Fig 17-24). The morphological features in TAM are identical to myeloid leukemia associated with DS and may often show dysplastic features in the bone marrow as seen in ML-DS. Blasts for both TAM and ML-DS show antigen expression characteristic of megakaryoblasts. Megakaryoblastic leukemic cells typically express CD34, CD117, CD13, CD33, HLA-DR, CD4 (dim), CD41, CD42, CD110 (TPOR), ILR3, CD36, CD61, and CD71, while lacking MPO, CD15, CD14, and glycophorin-A.¹³ In addition to trisomy 21, both TAM and ML-DS show *GATA1* mutations in blast cells; however, ML-DS will acquire additional mutations that lead to a progression into acute leukemia. Genetic abnormalities associated with the progression into acute leukemia include trisomy 8 and mutations in *CTCF*, *EZH2*, *KANSL1*, *JAK2*, *JAK3*, *MPL*, *SH2B3*, and *RAS* pathway.¹³

It is important to note that children with DS are also affected by both acute lymphoblastic leukemia and other acute myeloid leukemias, as the overall increased risk of leukemia applies to all subtypes of acute leukemia. In these cases, the leukemia should be classified based on the criteria for the leukemic disorders not associated with DS.

CRITICAL THINKING QUESTION

17-5 Are there exceptions to the WHO 20% rule?

Acute Lymphoblastic Leukemia/Lymphoma (ALL/LBL)

Acute lymphoblastic leukemia/lymphoblastic lymphoma (ALL/LBL) is the most common childhood malignancy. Approximately 75% of leukemias among children and teens are acute lymphocytic leukemia. Historically, ALL was universally fatal, but today an estimated 90% of newly

diagnosed children with ALL are cured.³⁶ Cases of ALL are rarely seen in adults, but the treatment can be significantly less effective.

To fully appreciate the classification of ALL and other lymphoproliferative disorders (e.g., CLL, lymphoma, and multiple myeloma), it is important to understand lymphocyte ontogeny. Lymphoid leukemias and lymphomas are a clonal proliferation of lymphoid cells that have been “frozen” at a given stage of maturation, while typically retaining some features of their normally differentiated counterparts. In ALL, the malignant clone has an immunophenotype with features of an early lymphocyte or “lymphoblast,” typically expressing terminal deoxynucleotidyl transferase (TdT) and either B or T lymphocyte antigens.

Review of Lymphocyte Ontogeny

Lymphocytes originate from pluripotent stem cells that are present in the yolk sac, fetal liver, spleen, and bone marrow. At birth and into adulthood, the stem cells are normally found only in the bone marrow, where they respond to specific growth factors (hormone-like substances) that trigger their commitment toward B- or T-lymphocyte differentiation. The microenvironment of these developing cells plays a critical role in their maturation: B cells develop in the bone marrow (bursa-equivalent tissue), whereas T cells develop in the thymus (from committed stem cells that have migrated there from the marrow). Lymphocyte maturation in these organs is antigen-independent. After the lymphocytes have matured, they migrate to the peripheral lymphoid organs, including the lymph nodes, spleen, and other lymphoid tissues. In these organs, the lymphocytes remain in a resting state until they are stimulated to undergo antigen-dependent development.

B-Lymphocyte Development

Early B-cell maturation (antigen-independent) is divided into three stages: early pre-B cell, pre-B cell, and mature B cell (Fig. 17-25). These stages are indicated by their expression of TdT, surface markers (HLA-DR, CD10 [CALLA], CD19, CD20), and immunoglobulin (cytoplasmic or surface Ig).³⁷ The early pre-B cell is TdT positive and expresses HLA-DR, CD19, and usually the common acute lymphoblastic leukemia antigen, CD10 (CALLA). HLA-DR, a histocompatibility-related antigen, is expressed first, followed by CD19 and then CD10. CD19 is the most sensitive and specific surface marker for early B cells.³⁸ During this stage, the immunoglobulin genes begin to undergo structural rearrangement followed by the production of cytoplasmic μ (C μ)-heavy chain.³⁷ The presence of cytoplasmic μ distinguishes the pre-B cell from its predecessor, which otherwise has a similar phenotype. As the cell continues to mature, immunoglobulin light chains are produced, and IgM is assembled and inserted into the plasma membrane. This surface Ig (sIg) is the hallmark of the mature B cell, which no longer expresses TdT. Each B cell expresses only one type of Ig light chain, either kappa (κ) or lambda (λ). This feature is extremely helpful in identifying monoclonal proliferations of mature B cells, because a normal population of B cells will consist of a mix of κ and λ light chain

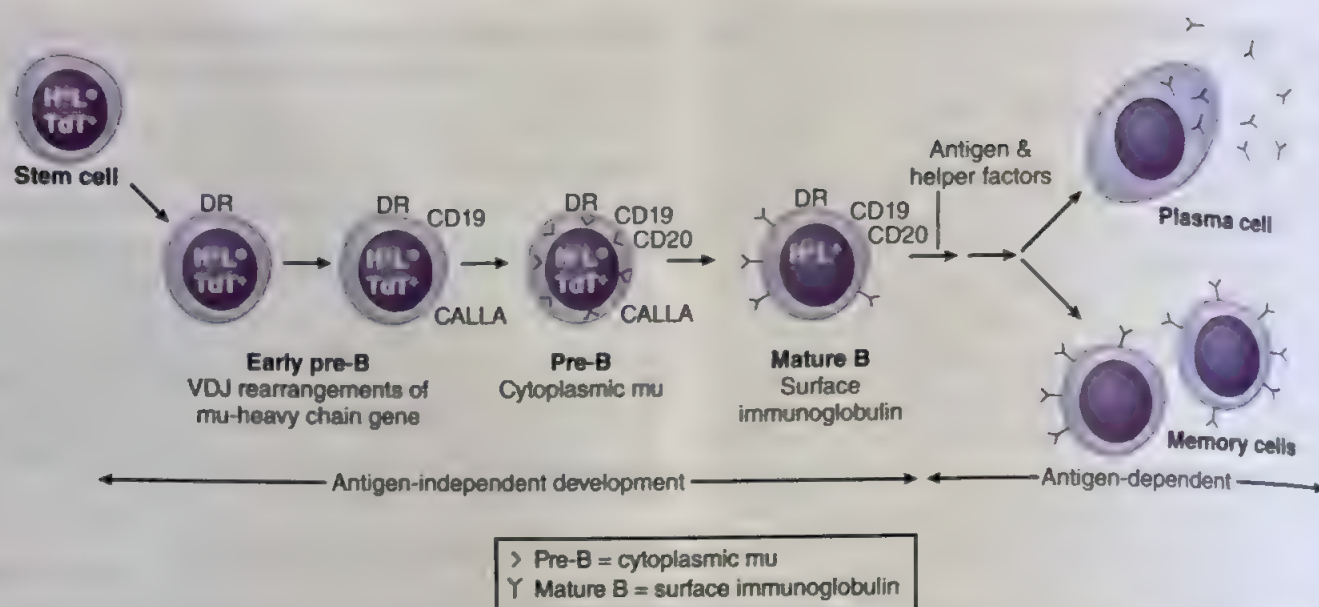


FIGURE 17-25 B-cell development. Heavy chain (H) and light chain (L) are designated as H⁰, L⁰ if in embryonic form, and H⁺, L⁺ if rearranged. > = cytoplasmic mu; Y = immunoglobulin.

expressing B cells. If all the B cells in a population express, κ for example, the population is likely clonal, because polyclonal B cells would be a mix of some B cells expressing λ along with others expressing κ .

Immunoglobulin genes are rearranged in a unique process that is normally limited to cells committed to B-cell differentiation. The Ig genes are composed of discontinuous segments of minigene families that, when productively rearranged, encode for the heavy chain and the κ or λ light chains.

ADVANCED CONTENT

The heavy chain gene (on chromosome 14) is composed of four minigene families including the variable (VH), diversity (DH), joining (JH), and constant (CH) regions. The CH region has separate DNA sequences that encode for the different Ig isotypes including mu (μ), delta (δ), gamma (γ), alpha (α), and epsilon (ϵ). The V, D, and J regions are the first to undergo rearrangement, forming a VDJ complex. In this process, intervening sequences (introns) are excised, and the V, D, and J regions are spliced together. Messenger RNA is transcribed from this VDJ complex along with DNA sequences downstream from it, including an intron and the constant μ (C μ) region. The mRNA itself is then spliced to bring the VDJ complex next to the C μ region, creating a template for cytoplasmic μ -heavy chain synthesis. This process is closely followed by a similar rearrangement of the κ gene (on chromosome 2), which if unsuccessful, is in turn followed by rearrangement of the λ gene (on chromosome 22). As B-cell development continues, the heavy chain may undergo additional rearrangements in the CH region, which initiate an isotype switch from μ to δ .³⁷ Both IgM and IgD are expressed on the surface of the majority of mature B cells (Fig. 17-26).

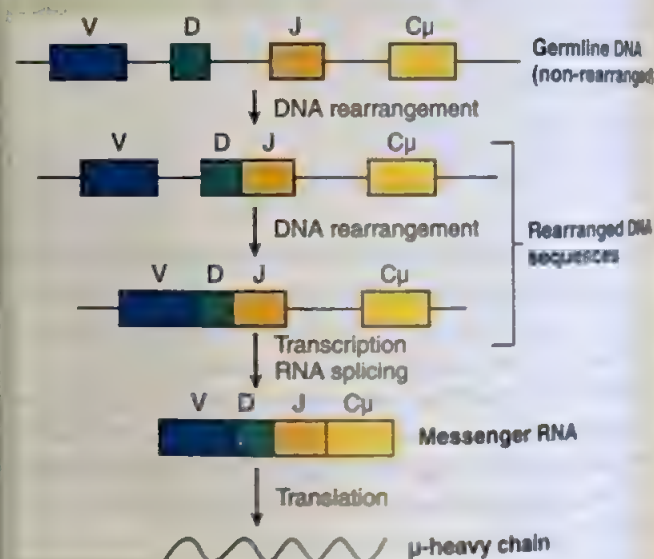


FIGURE 17-26 Schematic of immunoglobulin μ -heavy chain gene rearrangement. The variable (V), diversity (D), and joining (J) regions of germline DNA are linked through rearrangement and loss of intervening sequences. The VDJ complex, intervening sequences, and a constant (C μ) region are then transcribed. The resulting RNA is spliced, linking the VDJ and C μ regions and creating a template for the Ig μ -heavy chain. (Lines between blocks represent intervening sequences.)

After maturation in the bone marrow, B cells circulate through the blood to the peripheral lymphoid organs, where they remain in a resting state until stimulated by specific antigens to undergo further development. Activated B cells undergo clonal expansion, producing daughter cells with the same antibody idiotype (antigen-binding region). Some daughter cells become memory cells and regain the same mature B-cell morphology and phenotype, while others continue development toward a short-lived antibody-secreting cell—the plasma cell. During this final development, the heavy chain may undergo another isotype switch to IgG, IgA,

or IgE. The plasma cell produces large quantities of Ig and is characterized by a high concentration of cytoplasmic Ig.

T-Lymphocyte Development

In the past, T cells were identified by incubating lymphocytes with sheep red blood cells and observing for E-rosette formation (Fig. 17-27). Now, with the availability of monoclonal antibodies, T cells are identified and subclassified via immunological reagents. T-cell development (antigen-independent) in the thymus is divided into three main stages: stage I, early thymocyte; stage II, common thymocyte; and stage III, mature thymocyte. Stages I and II occur in the thymic cortex, and the last stage occurs in the thymic medulla. Similar to early

B cells, thymocytes express TdT and unique surface markers (Fig. 17-28). CD7, which is present on early thymocytes, is one of the earliest T-cell markers to be expressed; it is also the most sensitive marker for T-cell ALL. Its expression is followed by that of CD2 and CD5.³⁸ As the thymocytes move into stage II of thymic development, they express CD1, a marker of common thymocytes, along with CD4 and CD8 coexpression. CD3 is the next marker to be expressed; it is usually absent or only weakly expressed at stage II, but it is fully expressed in the mature thymocyte (stage III). At this stage, CD1 and CD4 or CD8 are lost, giving the mature thymocyte a CD4 or CD8 phenotype.³⁹

ADVANCED CONTENT

During thymic maturation, the T cell synthesizes an antigen-receptor molecule called the T-cell receptor (TCR), which is closely associated with the CD3 molecule on the plasma membrane. Two TCR isotypes have been discovered, TCR- $\alpha\beta$ and TCR- $\gamma\delta$. The genes that encode for the α , β , γ , and δ polypeptides undergo rearrangement in a manner that parallels Ig gene rearrangements in the B cell. The TCR- β gene rearrangements (on chromosome 7) precede TCR- α rearrangements (on chromosome 14). Less is known about the γ and δ genes or about the function of the TCR- $\gamma\delta$, but it is clear that rearrangement of the γ gene precedes that of the α and β genes. The majority of mature T cells express the TCR- $\alpha\beta$ isotype.³⁹

FIGURE 17-27 E-rosette formation in a T cell.

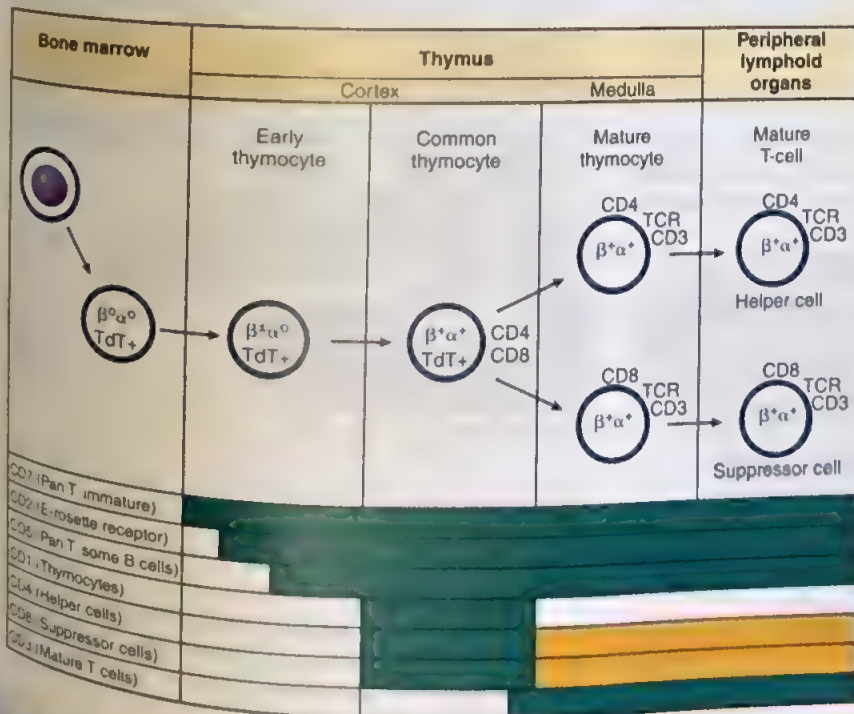


FIGURE 17-28 Normal T-cell maturation. T-cell receptor (TCR) β and α genes are designated as β^0 and α^0 if in the embryonic form, and β^+ and α^+ if rearranged. The colored blue bars indicate where the antigens are present in the stages of maturation. No color indicates the antigens are not present. Please note that the CD1 antigen disappears on mature thymocytes. The orange color indicates that as the cells mature they lose the expression of either CD4 or CD8, with only one of these antigens remaining on each mature thymocyte or T cell, and not both.

Clinical Findings

B-lymphoblastic leukemia/lymphoma (B-ALL) is primarily a disease of children. The clinical presentations of ALL are nonspecific, such as palpable liver and spleen, bone pain, fever, pallor, and bruising. Many patients have peripheral blood findings associated with neutropenia, anemia, and thrombocytopenia, with variable white counts. Symptoms result in infections, easy bruising or bleeding, and fatigue. Primary complaints may be bone pain, fever, and/or weight loss. T-lymphoblastic leukemia/lymphoma (T-ALL/LBL) is primarily a disease of adolescent males who present with lymphadenopathy or a mediastinal mass and occasional bone marrow involvement.

Morphology

The classification of ALL relies heavily on immunophenotypic, molecular, and cytogenetic characteristics of the clonal blast population. Morphologically, T-cell ALL/LBL is indistinguishable from B-cell ALL/LBL. The peripheral blood smear reveals two main types of lymphoblasts. First are large lymphoblasts with a moderate N:C ratio, multiple prominent nucleoli, and membrane irregularities. These cells may be easily confused with myeloblasts. The more prominent cell type is the small lymphoblast with high N:C ratio, condensed nuclear chromatin, and indistinct nucleoli. A few azurophilic granules in the cytoplasm may be seen, with an absence of Auer rods.

Historical Classification: FAB Classification of ALL

Historically, ALL was subtyped using the French-American-British (FAB) classification system. The morphology of these groups is evaluated on a bone marrow aspirate smear rather than peripheral blood.

The FAB classification system separates ALL into three morphological groups:

1. L1: Small, uniform lymphoblasts (Fig. 17-29)
2. L2: Large, pleomorphic lymphoblasts (Figs. 17-30 and 17-31)
3. L3: Burkitt's type (vacuolated and deeply basophilic cytoplasm in the lymphoblasts [Fig. 17-32])

As mentioned previously, genetic findings better predict prognosis. Today, most treatment centers have abandoned

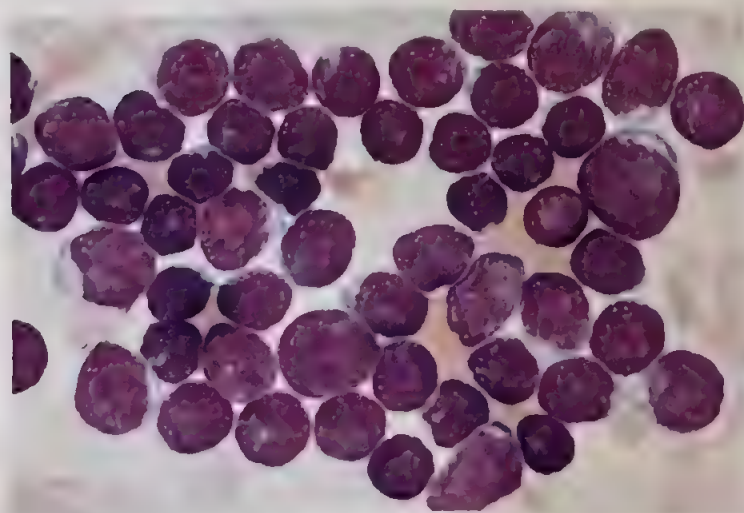


FIGURE 17-29 ALL, L1, bone marrow.

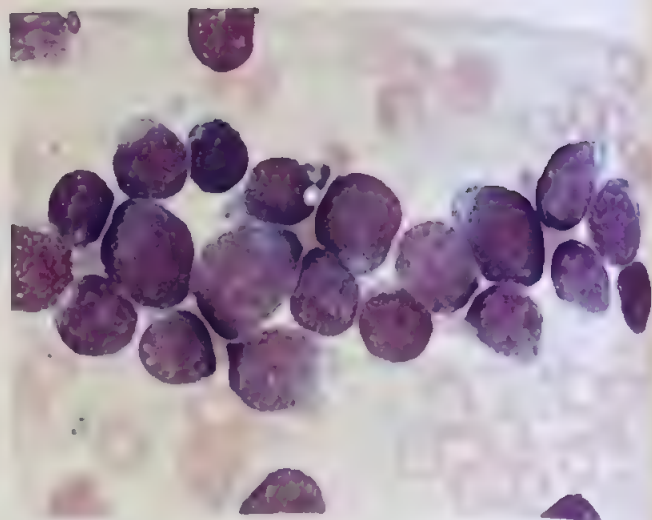


FIGURE 17-30 ALL, L2, bone marrow.

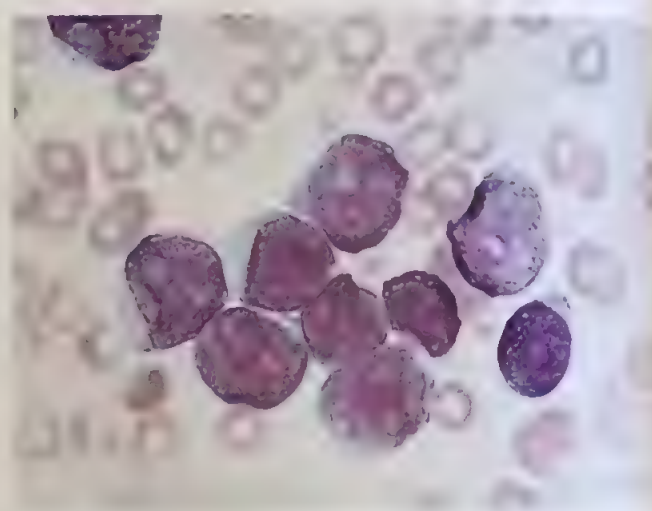


FIGURE 17-31 ALL, L2, peripheral blood.

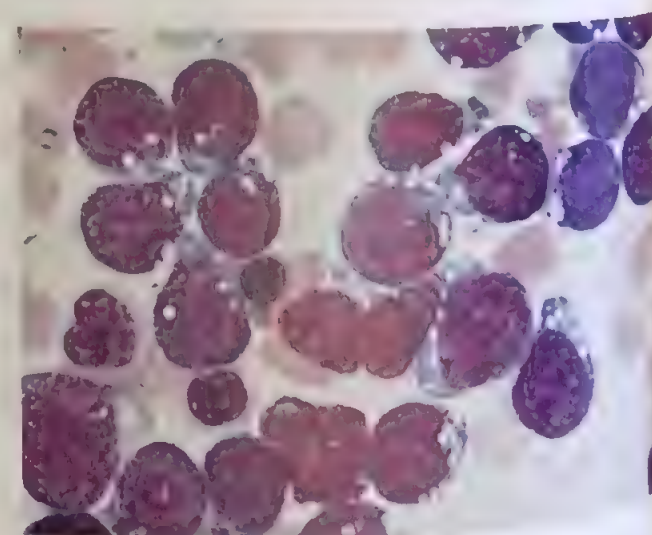


FIGURE 17-32 ALL, L3, bone marrow.

the FAB classification system of ALL and rely strictly on the World Health Organization (WHO) classification that includes immunophenotypic, cytogenetic, and molecular findings in therapeutic decisions.

World Health Organization Classification of ALL
The WHO classification of ALL is based on whether the clonal lymphoblast population is of B- or T-lymphocytic

lineage and seeks the associated recurrent cytogenetic abnormalities. The category, B-lymphoblastic leukemia/lymphoma not otherwise specified (NOS) is reserved for the B-ALL that do not have a recurrent cytogenetic abnormality. Categories of B-lymphoblast cytogenetic abnormalities are summarized in Table 17-11.

B Lymphoblastic Leukemia/Lymphoma, Not Otherwise Specified

B lymphoblastic leukemia/lymphoma, NOS was often subdivided into various stages based on the degree of maturation before the WHO classification system. The former classification distinguished between early pro-B-ALL (earliest), common B-ALL (intermediate), and pre-B-ALL (most mature) using the blast immunophenotype. B lymphoblastic leukemia/lymphoma is seen predominantly in the pediatric age group, although it may occur at any age. Its peak incidence is between the ages of 3 and 5 years, with 75% of cases presenting in children younger than 6 years old.⁴⁰

Patients with B-cell ALL/LBL show bone marrow involvement and often peripheral blood involvement as well (B-ALL). Extramedullary involvement of the CNS, lymph nodes, spleen, liver, and testes can be present. Occasionally, B-ALL/LBL can present with primary involvement of lymph nodes; however, there is typically some degree of leukemic involvement, which makes the distinction between ALL and LBL moot.

The term LBL is typically reserved for cases lacking or with minimal leukemic blasts identified. It is also uncommon for these patients to exhibit a markedly elevated white blood cell count (greater than $100 \times 10^9/L$). Historically, the two most important clinical predictors of how a patient would respond to treatment were age and white blood cell count at diagnosis; with age 10 or greater, age younger than 1 year, and WBC count greater than $30 \times 10^9/L$ predictive of the worse outcomes.⁴¹ Additional adverse prognostic and predictive factors include slow response to initial therapy (by morphological assessment of bone marrow blood), presence of minimal residual disease after therapy, and involvement of the central nervous system. While therapy decisions are still guided by clinical presentation, other risk factors such as the genetic characteristics of the lymphoblasts have become increasingly more important in determining prognosis and treatment, which are discussed later.

Flow cytometry is the clinical mainstay for diagnosing B-cell ALL. Lymphoblasts in B lymphoblastic leukemia/lymphoma show absent to dim CD45 expression and are typically positive for B-cell markers (CD19, cytoplasmic CD79a, cytoplasmic/surface CD22, PAX5), as well as CD10 and TdT. Expression of CD20 and CD34 is variable. Occasionally, expression of myeloid markers such as CD13 and CD33 may be expressed. The degree of differentiation can be determined by flow cytometry. For example, the earliest stage of B-ALL (pro-B-cell ALL) expresses HLA-DR, TdT, cytoplasmic CD79a, cytoplasmic CD22, and CD19. The intermediate stage, common B-cell lymphoblastic leukemia, expresses surface CD10. Pre-B-cell ALL (most mature) has the same surface phenotype but also expresses cytoplasmic

mu chain.² Although immunophenotype is no longer routinely used to distinguish between pro-B-cell and pre-B-cell ALL, it remains useful for identifying ALL and typically has clinical and genetic correlation.

For example, it was previously noted that the difference between children with pre-B-cell ALL ($C\mu$ present) and early pre-B-ALL ($C\mu$ absent) was important because the former was associated with a worse outcome. The distinction was based on blast immunophenotype. Both groups showed a high rate of achieving complete remission, but patients with pre-B-cell ALL appeared to have a shorter duration of remission.⁴² Subsequent analysis established that 20% to 30% of pre-B-ALL pediatric patients have a translocation of chromosomes 1 and 19 $t(1;19)$, which was considered an unfavorable prognosis before modern chemotherapy.⁴³ This rearrangement is not generally found in cases of early pre-B-cell ALL. Basing the distinction on cytogenetic immunophenotype, rather than blast immunophenotype, results in a more precise distinction, which contributed to targeted therapy effecting prognosis.

Pre B-cell ALL will typically show genetic abnormalities; however, it does not fall within the WHO classification system for B-ALL with recurrent genetic abnormalities. As mentioned previously, recurrent genetic abnormalities have become important for determining prognosis and guiding treatment.⁴¹ Genetic alterations associated with unique phenotypes or prognostic implications are classified as B lymphoblastic leukemia with recurrent genetic abnormalities.

B Lymphoblastic Leukemia/Lymphoma With Recurrent Genetic Abnormalities

B lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities are biologically distinct subtypes of A-BLL that have prognostic value.¹³

B Lymphoblastic Leukemia/Lymphoma With $t(9;22)$ ($q34.1;q11.2$);*BCR-ABL1*

B-ALL with $t(9;22)$ is associated with high-risk B-lineage ALL and is the most common chromosomal abnormality in adults. Up to 50% of patients above the age of 60 may show the Philadelphia chromosome.⁴³ This form of ALL was almost uniformly fatal in all age groups, but recent addition of tyrosine kinase inhibitor therapy to chemotherapy has shown increased long-term survival.⁴⁴ Blasts usually express CD10, CD19, and CD34, and many express some myeloid antigens like CD13 and CD33. Expression of CD25 is characteristic of B lymphoblastic leukemia with $t(9;22)$ in adults. The $t(9;22)$ translocation results in fusion of the *ABL* oncogene on chromosome 9 with the *BCR* gene on chromosome 22. The majority of childhood cases show a p190 *BCR-ABL1* fusion protein, while half of adult cases show p210 fusion protein.⁴⁵ No significant clinical difference is noted between the fusion proteins. Philadelphia chromosome may be detected by cytogenetics, next generation sequencing, RT-PCR, and FISH.

B Lymphoblastic Leukemia/Lymphoma With $t(v;11q23.3)$ *KMT2A (MLL) Rearranged*

B-ALL with a *MT24* rearrangement is a high-risk B-ALL that shows a translocation between *MT24* (*MLL*) at 11q23.3 with a wide variety of gene partners. The most common gene partner

with *KMT2A* is *AFF1* on chromosome 4q21; however, a multitude of other partners have been reported that do not affect the poor prognosis associated with this genetic abnormality.⁴⁵ This translocation is seen in the most common form of ALL in infancy and becomes more common with age in adulthood. Clinical presentation of the patient is typically associated with central nervous system involvement and a white blood cell count greater than $100 \times 10^9/L$.⁴⁵ The majority of acute leukemias with a *KMT2A* rearrangement show expression of CD15 and are CD10 negative by flow cytometry.⁴⁶ Translocations involving the *KMT2A* gene can be evaluated through cytogenetics, next generation sequencing, RT-PCR, and FISH.

B Lymphoblastic Leukemia/Lymphoma With t(12;21) (p13.2;q22.1);ETV6-RUNX1

Generally considered as a common translocation in precursor B-cell ALL in children, the t(12;21) occurs in about 25% of cases.⁴⁷ This subtype of ALL is associated with a favorable prognosis, especially when associated with other favorable risk factors. It is rarely found in infants or adults. The translocation results in fusion of the *ETV6* (*TEL*) gene on chromosome 12 and the *RUNX1* (*AML1*) gene on chromosome 21q. This gene fusion has a negative effect on transcription factors required for hematopoiesis.⁴⁸ Flow cytometric findings typically show strong expression of CD10 and absence of CD20. This translocation is detected by cytogenetics, next generation sequencing, RT-PCR, and FISH.

B Lymphoblastic Leukemia/Lymphoma With Hyperdiploidy

B-ALL with hyperdiploidy is common in children, accounting for 25% of cases in children under the age of 10. It is uncommonly seen in adults and infants. Hyperdiploidy is generally associated with a favorable prognosis. By definition, the blasts will show greater than 50 chromosomes without other recognized recurrent genetic alterations identified. Extra copies of chromosomes 21, X, 14, 10, 17, and 4 are the most commonly associated with this subtype of leukemia, while least seen with chromosomes 1, 2, and 3.⁴⁹ The most favorable prognostic group in this category is associated with gains of chromosomes 4, 16, and 17. Hyperdiploid B-ALL can be detected by cytogenetics, flow cytometric DNA index, and FISH studies.

B Lymphoblastic Leukemia/Lymphoma With Hypodiploidy

B-ALL with hypodiploidy is uncommon and associated with a poor prognosis in both adults and children. In this subtype of leukemia, the blasts show less than 46 chromosomes. B-ALL with hypodiploidy has been further subdivided into three categories: high hypodiploidy (42 to 45 chromosomes), low hypodiploidy (33 to 39 chromosomes), and near haploidy (23 to 29 chromosomes).⁵⁰ As chromosome number decreases, the prognosis becomes poorer; near haploidy has the worst prognosis.⁵¹ Hypodiploid B-ALL can be detected by cytogenetics, flow cytometric DNA index, and FISH studies.

B Lymphoblastic Leukemia/Lymphoma With t(5;14) (q31.1;q32.3);IL3-IGH

B-ALL with t(5;14) is rare and has been reported in both adults and children with an intermediate prognosis. Patient

may present clinically with a high white blood cell count due to eosinophilia. Many patients may develop clinical symptoms related to eosinophilia.⁵² The immunophenotype is like other B-ALL; however, small numbers of blasts in conjunction with eosinophilia are supportive of the diagnosis, because both peripheral blood and bone marrow may show low counts of lymphoblasts. The lymphoblasts show a translocation between *IL-3* and *IGH* gene resulting in eosinophilia.⁵³ The rearrangement can be detected by cytogenetics and FISH.

B Lymphoblastic Leukemia/Lymphoma With t(1;19) (q23;p13.3);TCF3-PBX1

B-ALL with t(1;19) accounts for approximately 6% of childhood cases. While it has been reported in adults, it is less commonly seen in older age groups. Historically, it was associated with a poor prognosis; however, modern chemotherapy has shown improved survival upgrading the subtype to an intermediate prognosis.⁵⁴ This translocation results in the fusion of the *PBX1* gene on chromosome 1 with the *TCF3* (*E2A*) gene on chromosome 19 and the formation of an abnormal fusion protein.⁵⁵ It is closely associated with strong CD10 and negative CD34 expression on the leukemic blasts. The rearrangement can be detected by RT-PCR methods and FISH in many cases.

B Lymphoblastic Leukemia/Lymphoma With BCR-ABL1-Like (Provisional Entity)

B lymphoblastic leukemia/lymphoma, *BCR-ABL1*-like is a heterogeneous group of leukemias lacking the *BCR-ABL1* translocation and is associated with an unfavorable prognosis. These leukemias show a similar gene expression profile to B-ALL with *BCR-ABL1* through translocations involving other tyrosine kinases, translocations involving cytokine receptor genes (*CRLF2*), and activating mutations or deletions in critical genes such as tyrosine kinase genes and Ras signaling pathways.^{56,57} Current data show half of these leukemias demonstrate a *CRLF2* rearrangement, which is most commonly seen in Hispanics, Native American ancestry, and children with Down syndrome. The prevalence is common and increases with age, accounting for nearly 25% of all adult B lymphoblastic leukemia, while making up only 10% of childhood B-ALL.⁵⁸ Clinical presentation and immunophenotype are similar to other subtypes of B-ALL; however, flow cytometry may be able to detect surface expression of the protein product of *CRLF2*. Detecting the diverse genetic abnormalities can be difficult because standard cytogenetics and FISH may not show the gene mutations of interest. A combination of next generation sequencing and whole transcriptome sequencing have been proposed with guidance from various diagnostic algorithms.⁵⁸

B Lymphoblastic Leukemia/Lymphoma With Intrachromosomal Amplification of Chromosome 21 (iAMP21)* (Provisional Entity)

B-cell lymphoblastic leukemia/lymphoma with *iAMP21* is uncommon, accounting for 2% of childhood B-ALL and is associated with a poorer prognosis. Patients typically are older children with a low white blood cell count. Cases of B-ALL with *iAMP21* show intrachromosomal amplification

of chromosome 21. Individuals with a Robertsonian translocation have a markedly increased risk of developing this subtype of leukemia, but etiology is not fully understood.¹¹ Given the poor prognosis and high risk of relapse associated with *iAMP21*, these children are typically treated with a more aggressive therapy course, which appears to overcome the adverse risk. No specific immunophenotype or morphological features are identified. Detection of *iAMP21* is typically performed by FISH with a probe for *RUNX1* that reveals additional copies of the gene.⁵⁹

(* means provisional change to WHO. May not have enough evidence for its own category.)

T-Lymphoblastic Leukemia/Lymphoma (T-ALL/LBL)

About 15% to 25% of patients with ALL have T-cell ALL.^{60,61} Historically, T-ALL cases were further categorized into one of the three stages of intrathymocyte development based on immunophenotype, but the prognostic significance of where the lymphoblasts are in these stages of development remains uncertain.^{13,60} Cytoplasmic CD3, CD2, and CD7 are expressed early in development. Surface CD3 is expressed after cytoplasmic CD3. CD7 is a very reliable marker of T-cell ALL, but CD2 and CD5 are also expressed in many cases.³⁸ A small subset of T-cell ALL cases express CD10 or *CALLA*, which is also seen on peripheral T-cell lymphoma and many B-ALLs.³⁸ Interestingly, CD4 and CD8 are occasionally coexpressed in T lymphoblasts, corresponding to a later stage of thymocyte development, but before selection of either CD4 or CD8 on mature T cells. Like the lymphoblasts of B-ALL, the lymphoblasts of T-cell ALL are TdT positive and are associated with both L1 and L2 morphology.⁶⁰

Patients with T-cell ALL often present with a mediastinal mass, a high white blood cell count (greater than $100 \times 10^9/L$ in 50% of cases), hepatosplenomegaly, and early meningeal involvement. Males are affected more often than females, and the disease occurs more often in older children. In the past, these patients generally had a poorer prognosis than those with B-ALL, but contemporary chemotherapy outcomes have steadily improved and approach those observed in B-ALL.⁶⁰

When patients present with a mediastinal mass, it may be difficult to distinguish T-cell ALL from lymphoblastic lymphoma. The morphological features of T-cell lymphoblastic lymphoma are similar to those of T-ALL. Although lymphoma is generally associated with a more mature immunophenotype, both the lymphoma and the leukemia are composed of TdT-positive lymphoblasts with evidence of T-cell lineage.³⁸ As in B-ALL/LBL, patients with T lymphoblastic lymphoma almost always develop some degree of bone marrow involvement, which renders their condition indistinguishable from T-cell ALL. Patients who first present with prominent marrow and peripheral blood involvement are usually diagnosed with T-cell ALL, even if they have concomitant mediastinal involvement. In contrast, a patient who presents with mediastinal involvement but little to no marrow (<20% to 25% lymphoblasts) or peripheral blood involvement is diagnosed with lymphoma.

T-cell lymphoblastic leukemia/lymphoma typically shows abnormal karyotypes and clonal rearrangements of T-cell

receptor genes.¹³ Chromosomal rearrangements, duplication/application of transcription factors, and gene deletions illuminate the genetic development of T-ALL. Recent advances in gene expression profiling suggest possible distinct genetic subgroups in the classification of T-ALL. Genetic subgroups based on specific translocations associated with distinct stages of thymic development have been proposed (aberrant expression of *TAL/LMO*, *TLX1*, *TLX3*, and *H0XA* genes).^{13,62} One of the more commonly involved genes, *TLX1*, has been associated with a favorable prognosis and can be seen in up to 30% of adult cases. In addition, impairment of *CDKN2A* and hyperactive *NOTCH1* are important oncogenetic events that have been identified in greater than 50% of T-ALL cases.⁶² Cases with *NOTCH1* mutation have been associated with shorter survival rates in adults. As advances in molecular techniques and accumulation of genomic data increase, opportunities for targeted therapy and prognostic groups for T-ALL/LBL are anticipated soon.

Early T-Cell Precursor Lymphoblastic Leukemia (Provisional Entity)

Early T-cell precursor acute lymphoblastic leukemia/lymphoma (ETP-ALL/LBL) is a recently recognized subtype of T-cell acute lymphoblastic leukemia/lymphoma that is relatively uncommon. This subtype of leukemia can be seen in 10% of childhood cases and 5% to 10% of adult cases. The morphology is similar to other forms of acute lymphoblastic leukemia, but the immunophenotype is distinct and shows an antigen expression pattern with one or more myeloid/stem cell markers.⁶³ Immunophenotypic findings for ETP-ALL/LBL typically showed expression of CD7 and cytoplasmic CD3, while lacking CD8, CD1a, and CD5. Expression of one or more myeloid stem cell markers is required for diagnosis (CD34, CD117, HLA-DR, CD13, CD33, CD11b, and/or CD65). It is important to note that expression of MPO is lacking, as MPO positivity would be supportive of a mixed phenotype acute leukemia.¹³ This subtype of leukemia highlights the fluidity between immature precursor T cells and myeloid lineage, supported by the fact that these leukemias show higher frequencies of mutations in *FLT3*, *RAS*, *IDH*, and *DNMT3A*, which are typically associated with myeloid leukemia.⁶³ T-cell genetic mutations such as *NOTCH1* and *CDKN2* are seen in lower frequencies.⁶³ Initially ETP-ALL/LBL represented a high-risk disease subtype with an overall poor long-term outcome; however, prognostic significance is currently still evolving.

Burkitt's Leukemia/Lymphoma (Mature B-CELL ALL)

Recall that the FAB classification scheme had three categories of ALL based on blast morphology. L3 lymphoblasts were Burkitt-like and morphologically had the features of lymphoblasts, so were classified as ALL. The WHO classification, with its emphasis on immunophenotype and genetics, does not include the FAB L3 subtype of ALL in its precursor B-cell ALL classification, because Burkitt-like lymphoblasts lack the immunophenotypic characteristics of an early B cell; namely, TdT is negative. The leukemic B cells in Burkitt's leukemia, previously called B-cell ALL, are derived from a

transformed or stimulated B cell; they are more mature B cells than the lymphoblasts in precursor B-ALL (see Chapter 22).

Childhood versus Adult ALL

The incidence and prognosis of ALL differs markedly with age. Disregarding genetics, recall that one of the most important clinical predictors of outcome in ALL is age, with patients 10 years old or older and infants younger than 1 year having a worse prognosis. Overall, about 90% of pediatric patients are alive and disease-free at 5 years compared with significantly lower rates in adults with ALL. For this reason, ALL may be considered in two subcategories: childhood ALL and adult ALL.

There are genetic and morphological differences between adult and pediatric ALL. L1 morphology occurs most frequently in the pediatric age group, whereas the L2 type tends to be seen in adults. The $t(9;22)$, a poor-prognosis translocation also known as the Philadelphia chromosome, has a significantly greater frequency in adult precursor B-cell ALL.⁶⁰ B-ALL with *KMT2A* rearranged are more commonly seen in infant B-ALL patients and largely account for the disappointing prognosis for ALL in patients less than 1 year old.⁶⁴ The most common cytogenetic abnormality in pediatric ALL is $t(12;21)$, seen in about 25% of pediatric cases and associated with a favorable prognosis.

Many patients diagnosed with ALL will achieve a complete remission, and many are potentially cured.⁶⁵ Unfortunately, some patients relapse or eventually die of their disease. The therapy used to achieve a cure has toxic effects that are a special concern in growing and developing children. To select the patients who will have an optimal response to less aggressive therapy, investigators have identified prognostic factors and defined clinical risk categories. These tools allow clinicians to better select the type and intensity of therapy to match each patient's risk of relapse most optimally against long-term sequelae of treatment. To date, indicators of a poor prognosis include older age, a high white blood cell count (more than $20 \times 10^9/L$), and particular genetic abnormalities, such as $t(9;22)$ *BCR-ABL1*, *KMT2A* rearrangement, and hypodiploidy.^{13,66} T-cell or mature B-cell phenotypes, often showing L2- and L3-type morphology, have also been associated with less favorable prognosis.¹³ Children with a low white blood cell count and certain genetic abnormalities such as $t(12;21)$ *TEL-AML1* and hyperdiploid lymphoblasts (more than 50 chromosomes) have a more favorable prognosis.^{13,66}

Acute Leukemias of Ambiguous Lineage

The advent of immunophenotyping, molecular, and genetic techniques has resulted in the understanding that some acute leukemias lack clear lineage but show characteristics of both myeloid and lymphoid, or rarely both B and T lymphoid lineages.⁶⁷ Diagnosis of ambiguous lineage leukemias is established through flow cytometric immunophenotyping of the acute leukemia, resulting in fewer than 2% to 5% of cases falling into the ambiguous lineage category.⁶⁸ Acute leukemias of ambiguous lineage generally have a poor prognosis. They can occur in children or adults but are more commonly seen in adults.⁶⁹ Ambiguous lineage leukemias

should be distinguished from AML with aberrant lymphoid antigen expression or ALL with one or two myeloid antigens. Leukemia subtypes for ambiguous lineage leukemias include biphenotypic and bilineal. Both biphenotypic and bilineal ambiguous leukemias are currently referred to as mixed phenotype acute leukemias (MPAL). Biphenotypic leukemias show lymphoid and myeloid antigens coexpressed by a single population of leukemic cells, while bilineal leukemia shows separate blast populations with distinct lineage-specific phenotypes.⁷⁰ Biphenotypic leukemias have been reported to evolve immunophenotypic expression over time with the possibility of developing two separate lineage-specific blast populations (bilineal). In addition, MPAL can relapse as pure AML or ALL posttreatment. Criteria for assigning more than one lineage to single blast population are recommended based on the immunophenotypic findings of the blasts. The most important lineage-specific antigens are CD19 expression with cytoplasmic CD79 α , cytoplasmic CD22 or CD10 expression for B-lymphoid lineages; cytoplasmic CD3, or less commonly surface CD3, or IHC for polyclonal anti-CD3 for T-lymphoid lineages; and myeloperoxidase (MPO) or monocytic differentiation for myeloid lineages.¹³ Current classification by the WHO further defines the ambiguous lineage leukemias based on the lineage specific markers that may be expressed (mixed phenotype acute leukemias) or complete lack of lineage specific markers (undifferentiated acute leukemia).

Treatment in ambiguous leukemia is evolving, but there are no current standard therapy guidelines. The majority of ambiguous lineage leukemias are treated as ALL in children and adults, while AML therapy is used in cases lacking CD19 or other lymphoid features. Recent studies have proposed treatment algorithms based on underlying genetic findings. For example, treatment as ALL for the genetic abnormalities such as *BCR/ABL1*, *ETV6/RUNX1*, and *TCF3/PBX1*, while treating with AML therapy for *RUNX1/RUNX1T1*, *PML/RARA*, and *CBFB/MYH11* leukemias.

Acute Leukemia of Ambiguous Lineage, Not Otherwise Specified

Acute leukemias of ambiguous lineage, not otherwise specified, are a group of leukemias with expression of a combination of markers that do not allow further classification as either acute undifferentiated leukemia or mixed phenotype acute leukemia.¹³ This subtype of leukemia is rare and can occur in both children and adults. Acute leukemias of ambiguous lineage lack a unique immunophenotypic. An example of an acute leukemia with ambiguous lineage would include leukemic blasts that express T-cell-associated markers (CD7 and CD3) and myeloid-associated antigens (CD13 and CD33) and lack cytoplasmic CD3 and MPO expression.⁷¹ Dim or weak expression of markers with limited lineage specificity in combination with more definitive lineage specific markers would not support a diagnosis of ambiguous lineage. These cases should be classified along the lines of the definitive lineage specific markers of the blasts with aberrant expression of other markers. An example would include acute myeloid leukemia with aberrant expression of B- or T-cell markers.

The prognosis is generally considered to be poor, with acute myeloid leukemia therapy usually attempted.

The hallmark of these rare leukemias of ambiguous lineage is that the overall findings are insufficient to classify as myeloid or lymphoid or the antigen expression is truly ambiguous and shows significant coexpression across lineages.

Mixed Phenotype Acute Leukemia MPAL, Not Otherwise Specified [MPAL NOS]

This subcategory is characterized by leukemic blasts that show evidence of both T-cell and B-cell lineage, which is extremely rare. Given the rarity of these cases, there are no defining characteristics for clinical features, genetic abnormalities, prognosis, or therapy. The WHO notes the importance of maintaining strict criteria for determination of lineage as described to avoid overreporting. For example, T lymphoblastic leukemia with expression of CD79a and CD10 should not be considered mixed phenotype or evidence of B-cell differentiation, as these markers have been associated with T lymphoblastic leukemia. Very few cases of trilineage (myeloid, B-cell, and T-cell) leukemia have been recognized. No evidence to date of mixed B/T lineage with megakaryocytic or erythroid lineage has been identified.

Acute Undifferentiated Leukemia

Acute undifferentiated leukemia is a rare leukemia that shows no specific markers for either lymphoid or myeloid lineage. Despite the limited data, these leukemias are associated with a very poor prognosis and are often associated with genetic mutations (*BAALC*, *ERG*, and *MNI*) linked to adverse prognostic indicators in acute myeloid leukemia.¹³ Flow cytometric studies show these leukemias to lack T-cell and myeloid markers (cytoplasmic CD3 and MPO), as well as B-cell markers (cytoplasmic CD22, cytoplasmic CD79a, and strong CD19). Typical undifferentiated leukemias show blasts with an immunophenotype positive for HLA-DR and CD34, but no more than one marker for any lineage should be identified.¹³ Comprehensive immunophenotypic studies should be performed to rule out megakaryocytic, basophils, NK precursors, plasmacytoid dendritic cells, or nonhematopoietic tumors.¹³

Mixed Phenotype Acute Leukemia (MPAL) With t(9;22) (q34.1;q11.2); *BCR-ABL1*

Mixed phenotype acute leukemia with *BCR-ABL1* shows a population of blasts with translocation (9;22); *BCR-ABL1* rearrangement. While it is the most common genetic abnormality seen in mixed phenotype acute leukemia, this leukemia accounts for less than 1% of all acute leukemias and can be seen in both children and adults. Occasionally, morphological review can reveal a dimorphic blast population with both myeloblasts and lymphoblasts identified. The most common immunophenotype reported meets the criteria for both B-cell and myeloid lineage.¹³ All cases show Philadelphia chromosome by cytogenetics or *BCR/ABL1* translocation by FISH. The p190 fusion transcript is most reported, while the p210 fusion transcript should prompt further investigation for CML in mixed blast crisis.¹³ This type of leukemia is associated with a very poor prognosis, particularly for

adults. Recent studies have shown treatment with tyrosine kinase inhibitors as part of chemotherapy have improved patient outcomes.⁷¹

Mixed Phenotype Acute Leukemia With t(v;11q23.3); *KMT2A* Rearranged

Mixed phenotype acute leukemia with *KMT2A* rearrangement shows a population of blasts with a translocation involving *KMT2A* in combination with criteria to meet the definition of mixed phenotype acute leukemia. Morphological review typically shows a dimorphic population of blasts with lymphoblasts and myeloblasts/monoblasts identified. As in acute lymphoblastic leukemia with *KMT2A* translocations, the rearrangement of *KMT2A* most commonly involves chromosome 4 (partner gene: *AFF1*).¹³ Given that many acute lymphoblastic leukemias may express myeloid antigens, it is important to note that such cases would not be considered a mixed phenotype acute leukemia. Detection of the *KMT2A* rearrangement can be done by standard karyotyping, FISH, and PCR. Mixed phenotype acute leukemia with *KMT2A* is a rare form of leukemia, which is more commonly seen in children, especially infants.⁴⁰ These patients typically have a poor prognosis. Cases are usually treated as ALL, but treatment may differ based on the presence of myeloid markers.

MPAL, B/Myeloid, Not Otherwise Specified

Mixed phenotype acute leukemias, B/myeloid not otherwise specified, show expression of both B-cell and myeloid component. The B-cell component, as previously mentioned, is characterized by strong CD19 with either cytoplasmic CD79a, cytoplasmic CD22, or CD10. If the expression of CD19 is weak, then expression of two out of the three B-cell markers is required (cytoplasmic CD79a, cytoplasmic CD22, or CD10).¹³ The myeloid component should show expression of MPO by flow cytometry, immunohistochemistry, or cytochemistry. If MPO is lacking in the blast population, then monocytic differentiation with the expression of at least two monocytic markers (nonspecific esterase, CD11c, CD14, CD64 lysozyme) should be present.¹³ Typically, MPO-positive blasts will show other myeloid markers such as CD13, CD33, and CD117, while expression of mature B-cell markers is uncommon. Morphology may show a dimorphic population of blasts or show blasts with features similar to lymphoblastic leukemia. Most cases show genetic abnormalities such as structural abnormalities of chromosomes (chromosome 6, 12, and 7 have been reported) and numerical abnormalities including near tetraploidy.⁷² In addition, recent advances have shown a gene expression profile that is intermediate between acute lymphoblastic leukemia and acute myeloid leukemia, which raises the possibility of additional genetic subtypes within this category in the future. Mutations typically found in acute lymphoblastic leukemia and acute myeloid leukemia have been reported, such as *ASXL1*, *TET1/2*, *IDH1*, *IDH2*, *DNMT3A*, and *NOTCH1*.⁷³ Mixed phenotype acute leukemia with B myeloid lineage is rare, accounting for 1% of all leukemia cases. Both adults and children are affected, but it appears to be more common in adults. The prognosis is generally considered poor, given that many of the identified mutations and chromosomal alterations typically are associated with poorer

prognosis. Various combinations of treatment have been tried, but there are currently no standard guidelines.

MPAL, T/Myeloid, Not Otherwise Specified

Mixed phenotype acute leukemia, T/myeloid, not otherwise specified, demonstrates both T and myeloid lineage, but no recurrent genetic abnormalities have been defined to date. The T-cell lineage component is characterized by strong expression of cytoplasmic CD3 by flow cytometry.¹³ T-cell lineage can also be determined using polyclonal anti-CD3 antibody immunohistochemistry, but activated NK cells may show positive staining; thus IHC is not entirely specific for T-cell lineage.¹³ In addition, T-cell origin can also be characterized by surface CD3 expression, but this is quite rare for mixed phenotype acute leukemias and typically represents a separate population of T-cell lineage blasts.⁷⁴ The T-cell component of the blasts may also express other T-cell markers such as CD7, CD5, and CD2. Determination of myeloid lineage is the same as previously described, with MPO in the blast population as the hallmark of myeloid lineage. Myeloblasts and monoblasts commonly can express other myeloid-associated markers, such as CD13, CD33, and CD117. As with other mixed phenotype acute leukemias, MPL with T/myeloid lineage is rare and accounts for less than 1% of all leukemia cases in both adults and children.⁷⁵ The morphological features are similar to other mixed phenotype leukemias, with blasts lacking distinguishing features (as in acute lymphoblastic leukemia) or showing a dimorphic blast population. While clonal chromosomal abnormalities are typically identified, none are frequent enough to suggest specificity for this subtype of leukemia.¹³ This leukemia subtype is generally considered to be associated with a poor prognosis. Various combinations of chemotherapy have been attempted, but there are no standard recommendations to date.

Treatment of Acute Leukemia

Therapy for acute leukemia (AL) is among the most complex of any anticancer programs. The goals of therapy for AL are to eradicate the leukemic clone, reconstitute normal hematopoiesis, and prevent any emergence of resistant leukemic subclones. Antileukemic therapy generally consists of three distinct phases: induction, consolidation, and maintenance.^{76,77} Induction therapy is designed to attain complete remission as quickly as possible; its success is one of the best predictors of long-term disease-free survival. Remission occurs when the patient's blood counts return to normal and bone marrow samples show no morphological or genetic sign of disease. Induction therapy achieves a remission in more than 95% of children and in about 75% to 89% of adults.⁸⁰ Induction therapy can be very intense and last about 1 month.⁷⁷ After induction chemotherapy, the next step is typically consolidation chemotherapy, depending on the patient and type of leukemia. The goal of consolidation therapy is to reduce the number of disease cells left in the body. The drugs and doses used during consolidation therapy depend on the patient's risk factors. Consolidation therapy typically lasts for four to eight months.⁷⁷ Maintenance therapy usually starts after a patient stays in remission following induction

and consolidation therapy. The goal is to destroy any leukemic cells that may remain to maintain a disease-free state. Maintenance therapy is less intense than the other two phases and may last two to three years.⁷⁷

The specific drugs used, their timing, and dosages differ remarkably among different AL subtypes. Results of AL therapy have improved over the past 20 years, with current investigation focused on targeting molecular markers to develop therapies that are more patient- and disease-specific. One example of genetic alterations leading to targeted treatment includes acute promyelocytic leukemia with *PML-RARA* (APL). Early recognition of APL is crucial because of an increased risk for fatal hemorrhage secondary to disseminated intravascular coagulation; thus the appropriate treatment for APL is different from that employed for other types of AML with this risk in mind.⁷⁸ APL therapy is curative in most patients; anticipated cure rates can be as high as 90% depending on other prognostic factors.⁷⁹ The addition of all-*trans*-retinoic acid (ATRA) to AML chemotherapy increases the complete remission rate and dramatically reduces the relapse rate in APL patients.^{78,79,80} Another example of a targeted therapy is the use of imatinib, a tyrosine kinase inhibitor, as well as chemotherapy for Philadelphia chromosome-positive ALL. In addition to use of tyrosine kinase inhibitors and chemotherapy, treatment may include hematopoietic stem cell transplantation once molecular remission is achieved. This regimen has resulted in a considerable improvement in outcomes in this formerly very unfavorable subgroup of ALL patients without significantly increasing toxicity.⁸¹

Response to therapy for AL is measured by several parameters. A complete remission (CR) has traditionally been defined as normalization of peripheral blood counts and a return to normal bone marrow hematopoiesis. The blast percentage should be normal, and there should be no persistence of disease either morphologically or with flow cytometric analysis. Cytogenetic analysis is typically performed before and after therapy, and the absence of a previously identified genetic abnormalities is another important component of CR. Newer techniques, such as multiparametric flow cytometry and molecular methods, can detect low levels of disease below the level detected by morphological review, immunophenotyping, or standard karyotyping, which illustrates that the achievement of a molecular complete remission is the best measure of treatment efficacy in AL. Molecular remission is defined as a level of minimal residual disease (MRD) that is below the detection limit of PCR analysis, which is generally one blast in 10,000 (10^{-4}) or 100,000 (10^{-5}) normal bone marrow cells. Persistence of detectable disease results in significantly worse survival.⁸² The presence of MRD is an independent prognostic factor that can identify clinical outcomes, primary drug resistance, and other factors that play a role in therapy response. MRD testing is being used in therapy of many AL to aid in decision making concerning the need for either maintenance or more aggressive therapy following induction. Patients with MRD detected by molecular methods, such as quantitative PCR, after consolidation therapy are candidates for more intensive therapy such as stem cell transplantation (SCIT).

CAR-T-cell therapy. Stem cell transplantation has been used for acute leukemia in the setting of certain genetic or chromosomal abnormalities, presence of minimal residual disease after induction, and relapse. Recent studies have shown that poor early minimal residual disease response in patients with acute lymphoblastic leukemia may benefit from allogeneic stem cell transplantation.⁶⁴

While chemotherapy, radiation, and stem cell transplant have been standard therapy for acute leukemias, newer immunotherapies are evolving rapidly with promising outcomes. One such example of immunotherapy is CAR-T-cell therapy

for relapsed/refractory acute lymphoblastic leukemia, in which a patient's own T cells are genetically engineered to recognize and kill cancer cells. Modification of the T cells occurs by insertion of genes that encode for tumor-specific chimeric antigen receptors (CAR), for example, CD19 for B-ALL. These modified T cells are subsequently infused into the patient and have shown significantly improved, even free, survival rates for patients with B-ALL. The integration of genetic markers into treatment schema is evolving rapidly, with new observations providing pivotal information about disease mechanisms and potential new drug targets.

SUMMARY CHART

- Acute leukemia tends to present with a sudden clinical onset and rapidly fatal course if untreated.
- Chronic leukemia has a more insidious onset and an indolent clinical course.
- The neoplastic cells in acute leukemia are immature (blasts, early myeloid cells), whereas the neoplastic cells in chronic leukemias are more mature.
- A diagnosis of acute leukemia is made using current WHO criteria by the presence of more than 20% blast cells in bone marrow aspirate smears or peripheral blood.
- Acute leukemia is divided into two basic categories based on cell lineage: myeloid and lymphoid.
- The distinction is critically important because the treatment and prognosis are different.
- Myeloblasts are usually large in size and have fine chromatin and prominent nucleoli. Lineage is confirmed by cytochemical staining and immunophenotyping.
- Lymphoblasts tend to be small- to medium-sized blasts with dense chromatin and less distinctive nucleoli. Lineage is confirmed by cytochemical staining and immunophenotyping.
- Auer rods are pathognomonic for myeloblasts in AML.
- Using cytochemical stains, myeloblasts are positive for myeloperoxidase (MPO) and Sudan black B.
- Nonspecific esterase (NSE) is positive in blasts with monocytic differentiation.
- Flow cytometry is an extremely useful tool for analyzing cell surface and cytoplasmic antigen expression in leukemia blasts and can be effective in differentiation AML, B-ALL, and T-ALL, as well as monitoring residual disease.
- Cytogenetic analyses (including karyotype and FISH) of leukemic cells can often identify chromosomal abnormalities, which can be critical in directing therapeutic decisions.
- Molecular diagnostic studies are becoming increasingly important and are used to confirm a suspected chromosomal abnormality, identify mutations that can affect prognosis and treatment decision, and monitor minimal residual disease following treatment or bone marrow transplantation.

CASE STUDY 17-1

A 42-year-old woman presented with a 2-month history of fatigue and weakness and a 2-week history of a sore throat. She reported a 15-pound weight loss over the past month or two. One week before admission, she started antibiotics for her sore throat, but she reported little improvement; she subsequently developed a peritonsillar abscess. On admission, she was found to have an elevated white blood cell count with a large number of circulating blasts.

The physical examination of the patient showed an anxious, middle-aged woman whose vital signs were normal, except for a slightly elevated temperature (37.6°C). Her right tonsil was enlarged and inflamed. She had no adenopathy, and her liver and spleen were not palpable.

Laboratory studies were ordered, and the following results were reported: hematocrit, 19.5%; hemoglobin, 6.3 g/dL; platelets, $64 \times 10^9/L$; and white blood cell (WBC) count, $79.2 \times 10^9/L$. A peripheral blood smear was manually reviewed in the hematology laboratory, and the differential diagnosis included 80% blasts.

A bone marrow aspirate and biopsy were obtained, which both showed virtually total replacement of normal elements with sheets of poorly differentiated cells (Fig. 17-33). The aspirate smears showed sheets of blasts. The blasts were relatively large (15 to 20 μm) with abundant cytoplasm. The nuclei varied in shape from round to oval, and some were indented or folded, most had several distinct small nucleoli.

Continued

CASE STUDY 17-1—cont'd

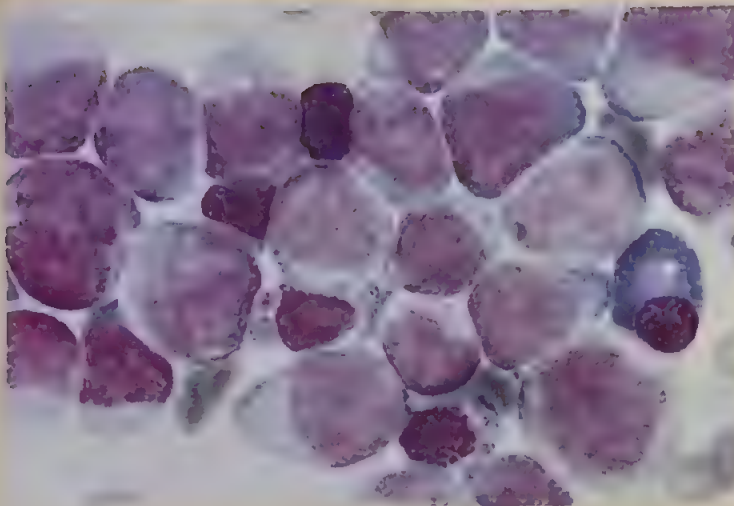


FIGURE 17-33 Case study bone marrow (AML with t(9;11)).

An occasional blast had delicate azurophilic granules, but this was the exception. No Auer rods were seen. The biopsy specimen was hypercellular, approaching 100% cellularity in some areas. The morphology of the aspirated cells was similar to those seen in the peripheral blood, except that fewer of the cells had folded nuclei.

A portion of the aspirate was analyzed using flow cytometry and showed an increase in blasts and immature monocytic cells. The blast immunophenotype was as follows: CD15, CD13, CD33, CD117, dim CD45, and HLA-DR.

FISH analysis demonstrated a chromosome rearrangement involving the *KMT2A* gene (11q23.3). Cytogenetics illustrated a translocation (9;11), which results in a rearrangement of the *MLLT3* gene at 9p21.3 with the *KMT2A* gene at 11q23 (Fig. 17-34).

Diagnosis: Acute Myeloid Leukemia with t(9;11); *KMT2A-MLLT3*

Follow-up: HLA matching was performed on the patient's brother and sister, but neither had a compatible tissue type. The possibility of bone marrow transplantation



FIGURE 17-34 Case study AML—karyotype with t(9;11).

was subsequently ruled out. The patient was placed on a standard protocol for acute myeloid leukemia.

QUESTIONS

1. What is the diagnosis and World Health Organization (WHO) classification for this patient?
2. Name the type of blast cell from the description given in the bone marrow aspirate and biopsy obtained.

ANSWERS

1. This represents a case of acute myeloid leukemia (AML) with recurrent genetic abnormalities. AML is the most common type of leukemia in adults. The specific WHO classification is AML with t(9;11); *KMT2A-MLLT3*.
The patient demonstrated the typical laboratory results for acute leukemia, namely anemia and thrombocytopenia. The white blood cell count in this patient was elevated at 79,200, and 80% blasts were reported in the differential.
2. The cells were monoblasts since the blasts were large with abundant cytoplasm and the nuclei were round to oval with some indented or folded.

CASE STUDY 17-2

A 32-year-old man presented to the ER complaining of fever, lethargy, and mucosal bleeding. He said that these symptoms began 5 to 6 days ago and that he had been in a general state of good health. The patient's past medical history was unremarkable. On physical exam, the patient appeared ill, pale, and in moderate acute distress. Numerous purpura were found on his upper and lower extremities, which the patient said had erupted over the past several days. No hepatosplenomegaly or enlarged lymph nodes were noted.

CBC and examination of the peripheral blood smear showed mild anemia with numerous schistocytes (red blood cell fragments). Severe thrombocytopenia and many erythrocyte fragments were also seen. Additional laboratory studies showed a markedly prolonged prothrombin time and decreased fibrinogen, indicating a profound coagulopathy.

A posterior iliac crest bone marrow aspirate and bone marrow biopsy were performed. The bone marrow biopsy and aspirate revealed a markedly hypercellular bone marrow

CASE STUDY 17-2—cont'd

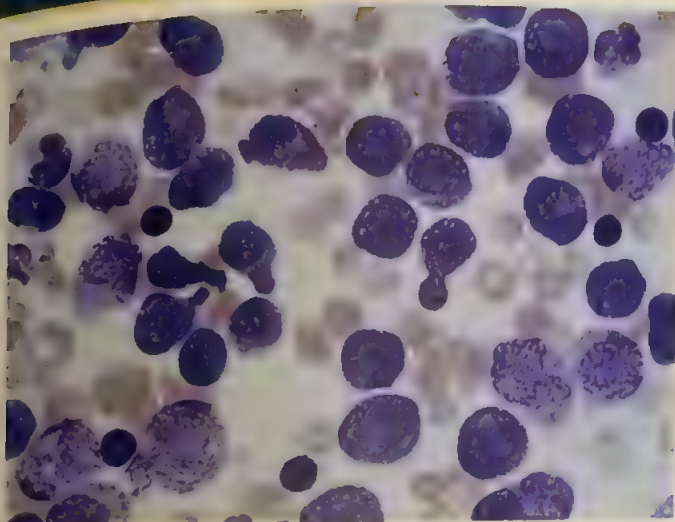


FIGURE 17-35 Case study APL with PML-RARA t(15;17).

with 80% promyelocytes, many with prominent azurophilic granulation and abnormal kidney bean-shaped nuclei. Some of the promyelocytes contained multiple Auer rods (Fig. 17-35). Cytochemical staining with myeloperoxidase (MPO) and Sudan black B showed strong staining in these promyelocytes. Flow cytometric analysis of bone marrow showed a myeloid population expressing CD13, bright CD33, CD45(dim), CD117, and no HLA-DR or CD34. Interphase FISH studies showed 91% of cells positive for the PML-RARA fusion.

QUESTIONS

1. What is the predominant leukemic cell in the bone marrow aspirate and why?
2. What is the diagnosis and World Health Organization (WHO) classification for this patient and why?
3. What other type of hemorrhagic manifestations can occur in APL?
4. What is the PML-RARA fusion in APL and what does it represent?
5. Describe the microgranular variant of APL with PML-RARA.
6. How is APL with t(15;17) treated?

ANSWERS:

1. The predominant cell is a promyelocyte since it had abnormal kidney bean-shaped nuclei, many with

prominent azurophilic granules. Some of the cells contained multiple Auer rods. Strong staining with myeloperoxidase and Sudan black B was observed in the promyelocytes.

2. This patient presents with an acute promyelocytic leukemia (APL) with PML-RARA. It is under the classification of acute myeloid leukemia with recurrent genetic abnormalities in the World Health Organization classification. The patient presents with the typical hemorrhagic manifestations of APL with numerous purpura on his upper and lower extremities. The CBC confirmed an anemia with numerous schistocytes on the peripheral blood smear and thrombocytopenia. The patient also had a prolonged prothrombin time and decreased fibrinogen, indicating a coagulopathy. The abnormal promyelocytes are rich in thromboplastic substances that, if released, trigger disseminated intravascular coagulation (DIC).
3. Bleeding can include petechiae, small ecchymoses, hematuria, bleeding from venipuncture and bone marrow sites, CNS, and pulmonary hemorrhages.
4. The PML-RARA represents a translocation that results in the fusion of a transcription factor called PML on chromosome 15 with the alpha (α)-retinoic acid receptor gene (RARA) on chromosome 17, giving rise to one of the most striking instances of genotype-phenotype correlation in pathology: the hybrid gene, PML-RARA. It is indicative of the t(15;17) translocation. Cytogenetics studies can confirm the translocation between the long arms of chromosomes 15 and 17.
5. In the microgranular variant of APL, the abnormal promyelocytes have a paucity of visible granules and the nuclei are typically bilobed. These atypical promyelocytes may be confused with monoblasts, but they are strongly positive with the myeloperoxidase stain.
6. All-transretinoic acid (ATRA) is the recommended therapy. ATRA is a vitamin A analogue that forces the promyelocytes through this stage of development. Due to the positive response to ATRA therapy, the prognosis of this leukemia is better than for other types of AML.

CASE STUDY 17-3

A 51-year-old female presented to her primary care physician with a history of fatigue and bone pain. On physical exam, the patient appeared ill, and no hepatosplenomegaly or enlarged lymph nodes were noted.

A CBC and peripheral blood smear were reviewed. The peripheral blood showed numerous blasts with a high nuclear to cytoplasmic ratio, dispersed nuclear chromatin, and prominent nucleoli. The CBC results are as follows:

CBC			
WBC	$41.0 \times 10^9/L$	MCV	84.3 fL
RBC	$4.98 \times 10^{12}/L$	MCH	26.3 pg
Hct	42.0%	MCHC	31.2 g/dL
Hgb	13.1 g/dL	Platelets	$174 \times 10^9/L$

A bone marrow examination was performed and revealed sheets of small blasts having scant cytoplasm and indistinct nucleoli (see Fig. 17-36). Flow cytometry studies of the bone marrow aspirate were performed and showed 63% blasts with the following immunophenotypic profile:

Flow Cytometry			
CD3, surface and cytoplasmic	Negative	CD10	Positive
CD5	Negative	CD19	Positive
CD7	Negative	CD20	Positive
		Spectrum	
CD34	Positive	CD22, surface and cytoplasmic	Positive with dim Surface
CD13	Negative	CD79a, cytoplasmic	Positive
CD33	Negative	MPO	Negative
CD117	Negative	TdT	Positive
CD25	Dim Positive	HLA-DR	Positive

A portion of the bone marrow aspirate was sent for FISH, cytogenetics, and next generation sequencing. The FISH study showed translocation (9;22) resulting in *BCR-ABL1* gene fusion in 88% of cells analyzed. A cytogenetics study showed a complex karyotype, which confirmed the translocation (9;22), resulting in *BCR-ABL1* gene fusion.

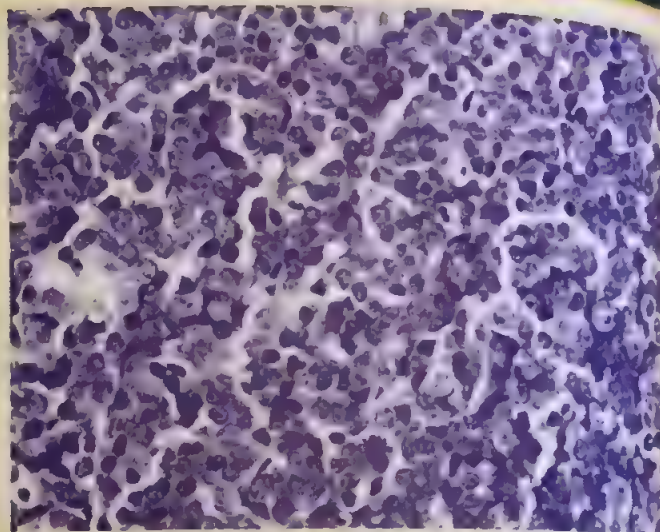


FIGURE 17-36 Case study B-ALL with BCR-ABL.

QUESTIONS

1. What is the predominant leukemic cell in the bone marrow aspirate and why?
2. What translocation and gene fusion is associated with this leukemia?
3. What is the diagnosis and World Health Organization (WHO) classification for this patient's leukemia?

ANSWERS

1. Lymphoblasts are the predominant cell since they were small blasts with a high nuclear to cytoplasmic ratio, dispersed nuclear chromatin and prominent nucleoli. Sheets of lymphoblasts were observed in the bone marrow examination, and the flow cytometry of the bone marrow aspirate demonstrated CD10, CD19, CD34, and CD25 characteristic of B lymphoblastic leukemia.
2. A translocation (9;22) resulting in the *BCR-ABL1* gene fusion was reported in 88% of the cells analyzed using the FISH technology. The cytogenetic study showed a karyotype, which confirmed the (9;22) translocation.
3. This patient's diagnosis and WHO classification is B-lymphoblastic leukemia/lymphoma with t(9;22); *BCR-ABL1*.

REVIEW QUESTIONS

1. How many blast cells in a bone marrow aspirate smear are necessary for a diagnosis of acute myeloid leukemia using WHO criteria?
 - a. 20%
 - b. 15%
 - c. 25%
 - d. 30%

2. What cytochemical stain is best for differentiating AML from ALL?
 - a. Alpha-naphthyl acetate
 - b. Nonspecific esterase
 - c. Myeloperoxidase
 - d. Periodic acid-Schiff

REVIEW QUESTIONS—cont'd

3. Which leukemia is most likely in a patient whose bone marrow shows abnormal proliferation of bizarre erythroid precursors?
 - a. Acute myelomonocytic leukemia
 - b. Acute megakaryocytic leukemia
 - c. Acute erythroid leukemia
 - d. Acute myeloid leukemia with minimal maturation
4. Which chromosome abnormality occurs in AML with *RUNX1-RUNX1T1*?
 - a. t(8;21)
 - b. t(9;22)
 - c. t(15;17)
 - d. t(1;19)
5. A 27-year-old woman presents with acute bleeding to the emergency department and is found to have a white count of $32.7 \times 10^9/L$ with pancytopenia: hematocrit 25%, platelet count $30 \times 10^9/L$, and absolute neutrophil count $0.8 \times 10^9/L$. She has a combination of 93% blasts and abnormal promyelocytes in her peripheral blood; some of these cells contain stacked Auer rods. What form of leukemia does she most likely have?
 - a. Acute myeloid with t(15;17)
 - b. Chronic myeloid leukemia
 - c. Acute monoblastic leukemia
 - d. Acute myeloid with t(8;21)
6. Which of the following is true about the prognostic implications of recurrent genetic findings seen in B-cell ALL?
 - a. Hypodiploidy is associated with a favorable prognosis.
 - b. t(12;21) *ETV6-RUNX1* is associated with a favorable prognosis.
 - c. *BCR-ABL1*-like is associated with a favorable prognosis.
 - d. Hyperdiploidy of greater than 50 chromosomes is associated with a poor prognosis.
7. Cells positive for CD2, CD3, CD5, CD7, and CD8 are characteristic of which of the following?
 - a. Precursor B-cell ALL
 - b. T-cell ALL
 - c. Pro B-cell ALL
 - d. Reactive lymphocytosis
8. The CALLA antigen found in ALL is also known as:
 - a. CD8
 - b. CD9
 - c. CD10
 - d. CD11
9. Which of the following is the most common genetic alteration observed in ALL?
 - a. t(1;19) *PBX1/E2A*
 - b. t(12;21) *ETV6-RUNX1*
 - c. t(9;22) *BCR-ABL1*
 - d. t(1;19) *TCF3-PBX1*
10. The purpose of induction therapy includes:
 - a. Attainment of remission state as quickly as possible
 - b. Elimination of tumor cells from tissues
 - c. Prevention of disease progression
 - d. Bone marrow transplantation

See answers at the back of this book.

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Myeloproliferative Neoplasms I

Chronic Myelogenous Leukemia

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CHAPTER OUTLINE

Chronic Myelogenous Leukemia

Etiology
Pathogenesis
Clinical Findings
Phases
Laboratory Testing and Results
Differential Diagnosis
Prognosis
Treatment

Atypical Chronic Myelogenous Leukemia

Chronic Neutrophilic Leukemia
Chronic Eosinophilic Leukemia, Not Otherwise Specified
Myeloproliferative Neoplasms, Unclassifiable
Summary Chart

Case Study 18-1

Case Study 18-2

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 18-1** State how chronic myelogenous leukemia is differentiated from the other myeloproliferative neoplasms, as classified by the World Health Organization (WHO).
- 18-2** List risk factors for the development of chronic myelogenous leukemia.
- 18-3** Explain the relationship between the Philadelphia chromosome, *t*(9;22), and the *BCR-ABL1* fusion gene.
- 18-4** Describe the role of the *BCR-ABL1* fusion gene and its protein product in the pathogenesis of chronic myelogenous leukemia.
- 18-5** Analyze the clinical presentation of chronic myelogenous leukemia, including the common presenting signs and symptoms.

- 18-6** List the three phases of chronic myelogenous leukemia, along with the diagnostic criteria for each.
- 18-7** Predict the common laboratory testing and results in chronic myelogenous leukemia.
- 18-8** Describe the cytogenetic analysis of chronic myelogenous leukemia, including the analytic techniques used and the advantages of each.
- 18-9** Evaluate the important differential diagnoses of chronic myelogenous leukemia.
- 18-10** Briefly describe the current treatment of chronic myelogenous leukemia.
- 18-11** List the factors most important in determining the prognosis of patients with chronic myelogenous leukemia.

Chronic myelogenous leukemia (CML) belongs in the group of myeloproliferative neoplasms and presents with marked proliferation of granulocytes in the bone marrow and peripheral blood. The natural progression of CML begins with the chronic phase, followed by the accelerated phase, and finally the blastic phase. The treatment and prognosis have improved greatly in recent years due to increased research and focus on tyrosine kinase inhibitors.

Myeloproliferative neoplasms (MPNs) describe a group of hematopoietic disorders that originate from the clonal expansion of a genetically altered hematopoietic pluripotent stem cell. The genetic alteration results in the excessive production of one or more “myeloid” (nonlymphoid) cell lines in the bone marrow, resulting in an increased expression of one or

more myeloid cells in the peripheral blood (i.e., granulocytes, erythrocytes, and platelets).¹ These disorders are chronic in nature with accelerated and/or acute phases. Classically, MPNs have included four clinical entities: chronic myelogenous leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). PV, ET, and PMF are presented and discussed in Chapter 19. However, the **World Health Organization (WHO)** has focused attention on the classification of MPNs in several publications and expanded the category to include seven subcategories by adding chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia (CEL), and MPN, unclassifiable (MPN-U).² The list of disorders classified under the MPN umbrella is found in Box 18-1.

BOX 18-1 WHO Classification of Myeloproliferative Neoplasms

- Chronic myelogenous leukemia [Ph chromosome, t(9;22) (q34;q11), *BCR-ABL1* positive]
- Chronic neutrophilic leukemia
- Chronic eosinophilic leukemia
- Polycythemia vera
- Primary myelofibrosis
- Essential thrombocythemia
- Myeloproliferative neoplasm, unclassifiable

Differentiation between MPNs is based upon identified molecular genetic alteration and predominant cell type and morphology in the bone marrow and peripheral blood.³

In CML, the bone marrow shows a granulocytic hyperplasia with a dominant increase of **granulocytes** (neutrophils, eosinophils, and basophils) in the peripheral blood.⁴ In PV, an erythroid hyperplasia in the bone marrow is accompanied by a corresponding increase of erythrocytes in the peripheral blood, whereas in ET, megakaryocytes are dominantly increased in the marrow and platelets in the peripheral blood. PMF would seem to be the exception because it is characterized by a decrease in most cells (pancytopenia) in the peripheral blood, especially late in its course. However, pre-PMF may show an increase in all cell types, at least before bone marrow fibrosis supervenes to cause pancytopenia.³

Myeloproliferative neoplasms are typically seen in adult patients and are rarely found in children. These disorders are chronic in nature, meaning that the progression is gradual and usually includes organ involvement. Patients may present with the following general clinical symptoms: pallor, malaise, weight loss, increased infections, weakness, splenomegaly, hemorrhage, and thrombosis.⁵

MPNs are the result of genetic alternations influencing the hematopoietic stem cell in the bone marrow. These molecular changes have numerous effects on the bone marrow environment. In addition to influencing cell line production from the affected stem cells, these genetic anomalies also influence the production of cytokines and/or how cells respond to corresponding growth factors.¹ It should be noted that MPNs can transform from one disorder into another, and all can lead to an acute leukemia, such as acute myeloid leukemia (AML) or acute lymphocytic leukemia (ALL). The transitions between disorders and overlapping characteristics can make exact classification of an individual case difficult (Fig. 18-1) The characteristics of the MPNs are outlined in Table 18-1.

CRITICAL THINKING QUESTION

18-1 Which hematopoietic stem cells are directly responsible for myeloid cell production?

See answers to all Critical Thinking Questions at the back of this book.

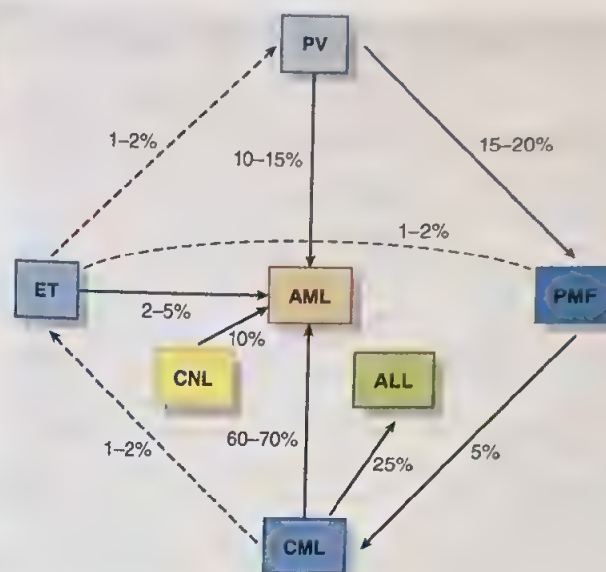


FIGURE 18-1 Relationship between the various myeloproliferative disorders. Frequencies of transitions between each of these bone marrow stem cell disorders are shown. AML = acute myelocytic leukemia; PV = polycythemia vera; PMF = primary myelofibrosis; CML = chronic myelogenous leukemia; CNL = chronic neutrophilic leukemia; ALL = acute lymphocytic leukemia. (A solid line indicates a strong relationship between disorders; a dashed line indicates a weak relationship.)

Chronic Myelogenous Leukemia

As stated in the introduction, CML, also referred to as chronic myeloid leukemia, is a chronic myeloproliferative disorder characterized by a marked increase of granulocytes in the peripheral blood, as well as a marked granulocytic hyperplasia of the bone marrow. CML was the first human disease in which the pathogenesis could be directly traced to a specific chromosomal abnormality.⁶ The Philadelphia (Ph) chromosome, first identified in 1960, results from a reciprocal translocation between the long arms of chromosomes 9 and 22 (written as t(9;22)), which can be identified in greater than 95% of patients with the typical characteristics of CML.^{7,8}

Since that time, much research has focused on the Ph chromosome and its role in the formation of the *BCR-ABL1* fusion protein, which is now thought to be the driver of CML.^{9,10} The Ph chromosome is characteristic of the disease but not solely diagnostic since it is also detected in cases of acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), and mixed-phenotype acute leukemia (MPAL).¹¹ Proper diagnosis of CML requires the identification of either the Ph chromosome and/or the *BCR-ABL1* gene and corresponding cellular morphology.⁹

Etiology

CML accounts for 20% of leukemia cases in adults, with approximately 8,450 estimated new cases in the United States in 2020 and an annual incidence of 1.9 cases per 100,000 population.¹² There is a slight male predominance (male to female ratio 1.5:1). CML is most frequently diagnosed in patients

TABLE 18-1 Characteristics of the Myeloproliferative Neoplasms

Subcategory	Chronic myelogenous leukemia (CML)	Polycythemia vera (PV)	Essential thrombocythemia (ET)	Primary myelofibrosis (PMF)	Chronic neutrophilic leukemia (CNL)	Chronic eosinophilic leukemia (CEL)
Dominant Cell Type	Granulocytes: neutrophils and basophils	Erythrocytes	Megakaryocytes	Neutrophils and megakaryocytes early, fibrosis late	Neutrophils	Eosinophils
WBC count	↑↑↑	↑↑	↑	Variable	↑↑	↑
Hemoglobin	N to ↓↓	↑↑↑	N to ↓	↓	N to ↓	N to ↓
Leukocyte alkaline phosphatase	↓	N to ↑	N to ↑	Variable	↑	N
Philadelphia chromosome	>95%	Absent	Absent	Absent	Absent	Absent
Major genetic alteration	<i>BCR/ABL1</i>	<i>JAK2</i>	<i>JAK2/CALR/MPL</i>	<i>JAK2/CALR/MPL</i>	<i>CSFR3R</i>	<i>Trisomy 8</i>

N = Normal; ↓ = slight decrease; ↓↓ = moderate decrease; ↑ = slight increase; ↑↑ = moderate increase; ↑↑↑ = marked increase.

aged 65 to 74, with the median age of diagnosis at 65.¹² It is unusual to find CML in children.

Risk factors for developing CML include exposure to ionizing radiation¹³ (seen in radiologists before the use of safety shielding techniques, patients treated with radiation therapy, and survivors of nuclear explosions); to cytotoxic drugs, especially alkylating agents; and to biologically active chemicals such as benzene.¹⁴ However, the vast majority of CML patients report none of the previous risk factors or exposures; the cause in more than 95% of CML cases is unknown. CML is not an inherited disease; rather it appears to be acquired, as suggested by the rarity of familial aggregations of CML.¹⁴

Pathogenesis

CML is a clonal stem cell disorder in which the abnormal clone is characterized by the presence of the Ph chromosome.⁹ The chromosomal translocation that creates the Ph chromosome is *t*(9;22), a reciprocal translocation between the long (q) arms of chromosome 9 and chromosome 22. A smaller piece of the long arm of chromosome 9 is broken off at band q34.1 and translocated to the long arm of chromosome 22; at the same time, a larger piece of the long arm of chromosome 22 is broken at band q11.21 and moved to chromosome 9. This results in a small, abnormal chromosome 22 (the Ph chromosome; Fig. 18-2).

The notation describing this translocation, *t*(9;22)(q34;q11), defines both the translocation and the specific break points in chromosomes 9 and 22. In the translocation, the *ABL1* gene, the cellular homologue of the Abelson murine leukemia virus oncogene from chromosome 9, is brought into contact with the *BCR* (break cluster region) gene on chromosome 22 (Fig. 18-3). The resulting chimeric gene, located on the Ph chromosome and referred to as the *BCR-ABL1* gene, is transcribed, producing a *BCR-ABL1* fusion protein that has enhanced tyrosine kinase activity.⁹ The *BCR-ABL1* fusion protein causes aberrant activation of several cellular signaling pathways and is considered a hallmark of CML.¹³ Over

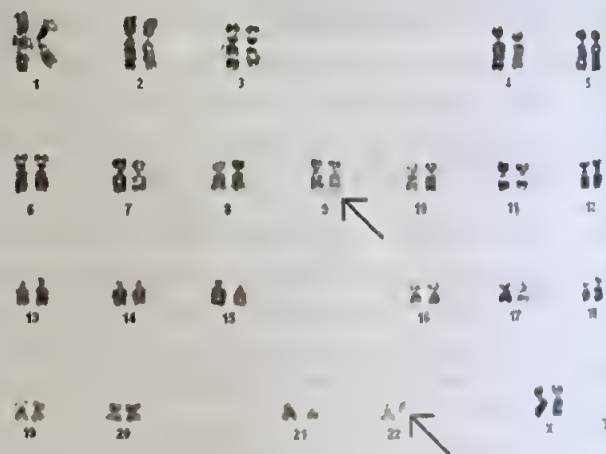


FIGURE 18-2 A metaphase spread from a patient with CML showing *t*(9;22). The abnormal chromosomes 9 and 22 are marked with arrows. The Philadelphia chromosome is the abnormal chromosome 22 on the right; the normal chromosome 22 is on the left.

95% of CML patients will be Ph chromosome positive,⁹ and 98% of patients will have a *BCR-ABL1* gene when studied via molecular techniques.¹¹ Those patients who do not have an identifiable *BCR-ABL1* gene are considered atypical CML, a separate disorder with a different prognosis and treatment (see Differential Diagnosis later).

The Ph chromosome is not specific for CML; it has also been found in cases of acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), and mixed-phenotype acute leukemia (MPAL).¹¹ This suggests that the original cell in which the mutation arose was a pleuripotent stem cell, before separation of the lymphoid and the myeloid lineages. It is the presence of the Ph chromosome carrying the *BCR-ABL1* gene that gives the abnormal CML clone its growth advantage over normal cells and allows them to replace the normal bone marrow elements.

All cases of leukemia appear to be maintained by a pool of self-renewing malignant cells. In CML, immature granulocytes fill the bone marrow and are released prematurely into the peripheral blood in various stages of maturation. On the molecular level, it has been established that it is the chimeric *BCR-ABL1* fusion protein that plays the central role in myeloid proliferation and transformation in CML. There is direct evidence from in vitro studies that the *BCR-ABL1* protein (p210^{BCR-ABL}) causes uncontrolled cell growth in hematopoietic cell lines.⁴ Effects of the *BCR-ABL1* fusion protein in the CML cells include increased proliferative capacity, a slight delay in maturation, and a lack of responsiveness to the normal regulators of growth (e.g., cytokines or the bone marrow microenvironment). The abnormal protein also seems to prevent apoptosis (normal programmed cell death) in the CML clone.⁴

ADVANCED CONTENT

Depending on the precise break point in the *BCR* gene, the *BCR-ABL1* fusion protein can vary in size from 190 kDa to 230 kDa.⁸ The most common rearrangement of the *BCR-ABL1* transcript shows alteration between exons 13 and 14 of *BCR* and exon 2 and 11 of *ABL1*, producing a 210-kDa protein with tyrosine kinase activity (called p210^{BCR-ABL}). Nearly all patients with typical CML in chronic phase have the 210-kDa protein. A subset of CML patients with a 230-kDa

protein may be misdiagnosed with CNL due to neutrophil predominance; however, identification of the Ph chromosome will properly identify a case of CML.⁹ Patients with Ph chromosome-positive acute lymphoblastic leukemia may present with either the 210-kDa protein or a 190-kDa protein.¹⁵ This suggests that not all Ph chromosomes or *BCR-ABL1* genes are identical, and also that fusion proteins of different sizes can be correlated with different clinical outcomes.

The genetics of CML can occasionally be even more complex. Approximately 5% of CML patients have the deleted portion of chromosome 22 translocated to other chromosomes, such as t(4;22), t(12;22), and t(19;22). These are simple variant Ph chromosome translocations, whereas other patients have complex variant translocations involving two or more chromosomes in addition to chromosome 22.¹¹ On occasion, the Ph chromosome may be "masked" by the presence of a larger piece of chromatin material translocated to the abnormal chromosome 22 from one of the other chromosomes involved in the rearrangement giving a normal-sized, but abnormal, chromosome 22. The CML clone is genetically unstable and may accumulate additional mutations as it progresses to accelerated and blastic phase, which complicates diagnosis and treatment options.

Clinical Findings

The signs and symptoms, phases, and laboratory findings for CML are distinctive.

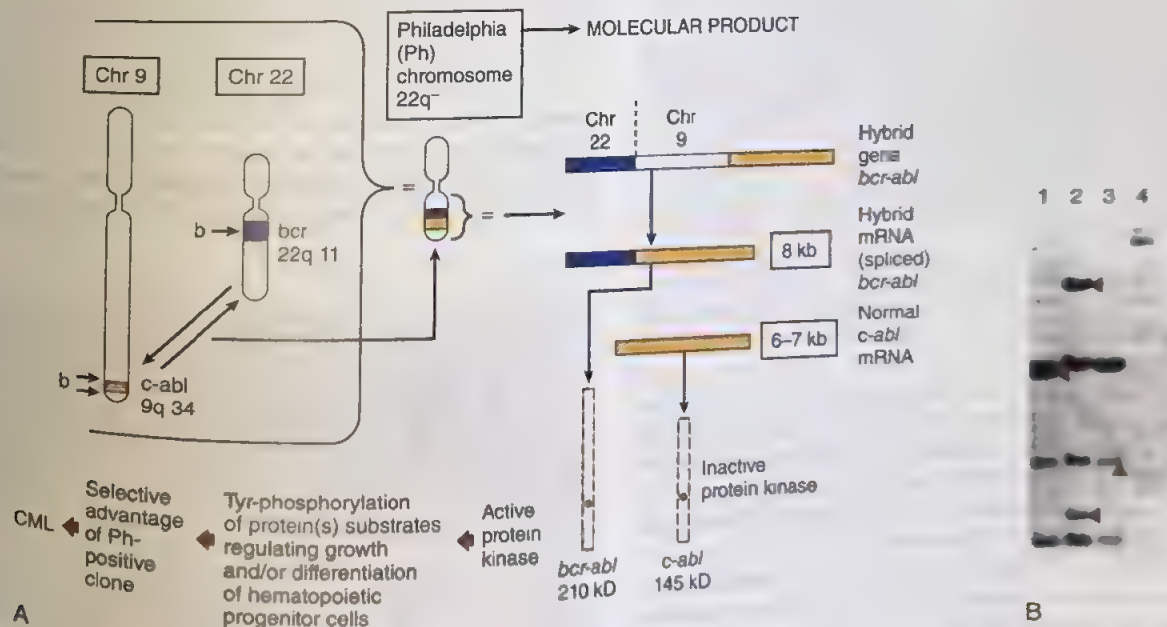


FIGURE 18-3 Molecular basis of the Philadelphia (Ph) chromosome. (A) Sequence of molecular and biochemical events involved in generating the Ph chromosome and its phenotypic consequences. (B) Southern blot analysis of DNA from CML cells analyzed with a *bcr* probe to show clonal rearrangements in the *bcr* region. Lane 1: Ph-positive CML DNA showing one rearranged band; lane 2: Ph-positive CML as in lane 1 but with a different break point in the *bcr* region; lane 3: Ph-negative leukemic cell DNA showing no rearranged *bcr*; and lane 4: molecular weight markers. (From Greaves MF. Cellular identification and markers. In Zucker-Franklin D, et al, editors. Atlas of Blood Cells. Milano, Italy: Lea & Febiger, Philadelphia: EE edi-ermes, 1988, p 43, with permission.)

Patients with CML may be asymptomatic or symptomatic at presentation. It is common for the disease to be discovered incidentally during a routine physical examination or routine hematologic evaluation of an asymptomatic patient.⁹ When symptoms do occur, the most common complaints are general malaise; fullness in the upper abdomen with early satiety, and loss of appetite related to **splenomegaly** (enlarged spleen) and **hepatomegaly** (enlarged liver); complaints resulting from a hypermetabolic state such as night sweats and weight loss; bone tenderness and aching related to marrow expansion; and complaints related to an accompanying anemia.⁹

Lymphadenopathy, which is rarely seen except late in the course of the disease, is associated with a poor prognosis. Some patients will have bleeding complications related to qualitative or quantitative platelet disorders. Rarely, patients may show manifestations of **leukostasis** (increased blood viscosity secondary to a very high WBC count) with vaso-occlusion, such as cerebrovascular accident, myocardial infarct, venous thrombosis, priapism, visual disturbances, and pulmonary insufficiency. In the later stages, patients may occasionally present showing effects of basophilia with increased serum histamine, including pruritus, diarrhea, and refractory peptic ulcer disease.

CRITICAL THINKING QUESTION

18-2 Which clinical symptoms can be attributed to anemia?

Phases

CML is characterized by three phases, which were significant for patient prognosis before the now standard treatment regime of tyrosine kinase inhibitor (TKI) therapy. The three phases are:

1. Chronic phase (CP)
2. Accelerated phase (AP)
3. Blastic phase (BC)

Most patients (95%) are diagnosed in the chronic phase, which is also referred to as the initial phase.¹⁶ Patient evaluation during this phase must include both peripheral blood and bone marrow analysis for cellular morphology, and the demonstration of the *BCR-ABL1* fusion gene, as well as other cytogenetic abnormalities. During this phase, the disease may remain stable for several years and is usually responsive to tyrosine kinase inhibitors (TKIs). Chronic phase CML patients can have a normal life expectancy when the optimal response is achieved by treatment.

Progression of CML to accelerated phase or blastic phase corresponds with a poorer prognosis, and usually occurs 3 to 5 years after onset, in the absence of therapy.⁴ The accelerated phase is heralded by a variety of signs and symptoms of disease progression that do not meet the criteria for the blastic phase. Associated worsening symptoms may include unexplained fevers, significant weight loss, progressive leukocytosis, worsening splenomegaly, requirement for higher doses

of myelosuppressive agents, bone and joint pain, bleeding, thrombosis, and infections.¹⁷ Patients in the accelerated and blastic phases display high genetic instability, which leads to the accumulation of TKI-resistant point mutations and limiting TKI effectiveness.¹⁸ Only 10% of CML patients will progress to the blastic phase (previously called blast crisis or blast transformation), which represents the conversion from CML to an aggressive form of acute leukemia that is difficult to treat. The corresponding leukemia can be myeloid, lymphocytic, or in rare cases both.⁴ Disease progression is defined by the blast count in the peripheral blood—chronic phase with $\leq 10\%$, accelerated phase with 10% to 20%, and blastic phase with $>20\%$ blasts.³ The criteria for diagnosing progression to accelerated phase and blastic phase are discussed in the paragraphs that follow.

Laboratory Testing and Results

The most important laboratory finding in the peripheral blood is an increased white blood cell count (frequently above $100,000/\mu\text{L}$), reflecting neutrophilic leukocytosis with basophilia (Fig. 18-4). Neutrophils vary in presentation and include virtually all maturation stage in the neutrophilic series, from segmented neutrophils to occasional blasts (fewer than 10%).³ The presence of a greater percentage of myelocytes than the more mature metamyelocytes is a typical finding (the “myelocyte bulge”).⁹ Most neutrophilic precursors appear morphologically normal, although occasional cells with pseudo-Pelger-Huët anomaly may be seen late in the disease (Fig. 18-5). Increased basophils are invariably seen in CML, such that if the absolute basophil count is not increased, a diagnosis of CML should be seriously questioned. Absolute eosinophilia is observed in 90% of cases, and increased monocyte counts are often seen. Thrombocytosis is present in about one half of cases and is usually of moderate degree (less than $1,000,000/\mu\text{L}$).⁹ Decreased platelet counts are uncommon, at least in the chronic phase.⁹ Micromegakaryocytes can be seen in circulation in as many as 25% of CML patients (Fig. 18-6). Normocytic anemia is commonly present, although occasional patients may have

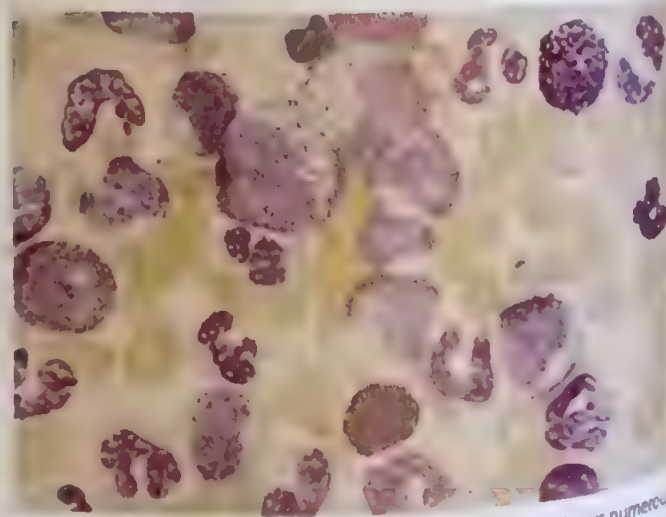


FIGURE 18-4 Peripheral blood from a patient with CML shows numerous mature neutrophils along with bands, metamyelocytes, and myelocytes. Note the two basophils at the upper right and bottom center.

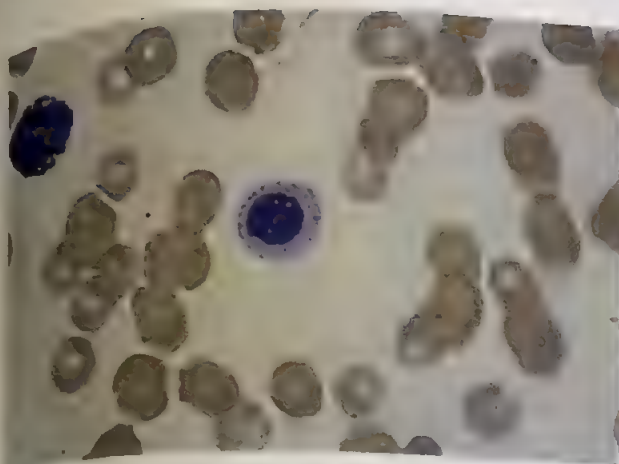


FIGURE 18-5 Pergeroid (pseudo-Pelger-Huët) neutrophil in CML. Note that the cytoplasm is mature and the nucleus is small and round with condensed chromatin. This cell can be mistaken for a myelocyte. A basophil is in the upper left corner.

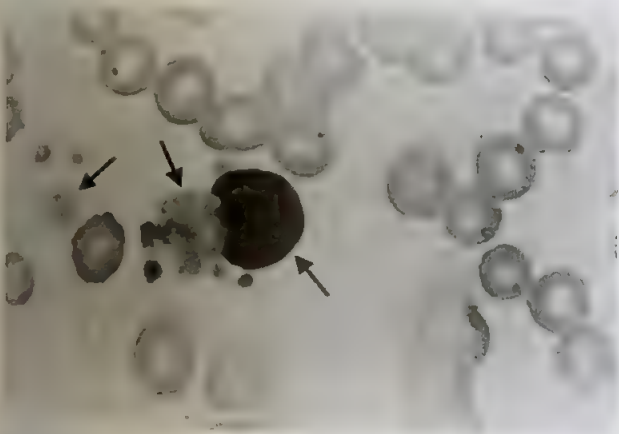


FIGURE 18-6 A micromegakaryocyte with giant platelets, from the peripheral blood of a patient with CML.

normal or even increased hemoglobin levels. The severity of anemia present is commonly proportional to the degree of leukocytosis.

A summary of the laboratory features of chronic myelogenous leukemia at diagnosis is provided in Table 18-2. With progression to the accelerated phase, patients may develop worsening anemia, worsening thrombocytosis or thrombocytopenia, and increasing peripheral blood basophils ($\geq 20\%$). There is also a shift to the more immature myeloid forms with an increasing number of blasts (10% to 20%). The blastic phase is heralded by an increase in blood or bone marrow blasts to more than 20%.

CRITICAL THINKING QUESTION

18-3 Which CBC result identifies normocytic anemia?

The bone marrow is hypercellular with a marked granulocytic hyperplasia (Fig. 18-7) with a typical myeloid-to-erythroid (M:E) ratio of 10:1 to 50:1. Maturation from blasts

TABLE 18-2 Laboratory Features at Presentation of Chronic Myelogenous Leukemia

Laboratory Test	Features
Peripheral Blood	<ul style="list-style-type: none"> • Neutrophilic leukocytosis with immature forms • Basophilia/eosinophilia • Thrombocytosis • Anemia • Blasts $< 10\%$ (in chronic phase) • Decreased leukocyte alkaline phosphatase (LAP) score • Increased lactate dehydrogenase • Increased uric acid • Increased vitamin B₁₂/transcobalamin
Bone Marrow	<ul style="list-style-type: none"> • Myeloid hyperplasia • Blasts $< 10\%$ (in chronic phase) • Minimal/no dysplasia • Increased megakaryocytes • Myelofibrosis (mild/moderate) • Monocytes usually $< 3\%$
Genetic Studies	<ul style="list-style-type: none"> • Philadelphia chromosome positive (95%) • BCR-ABL1 positive (98%)

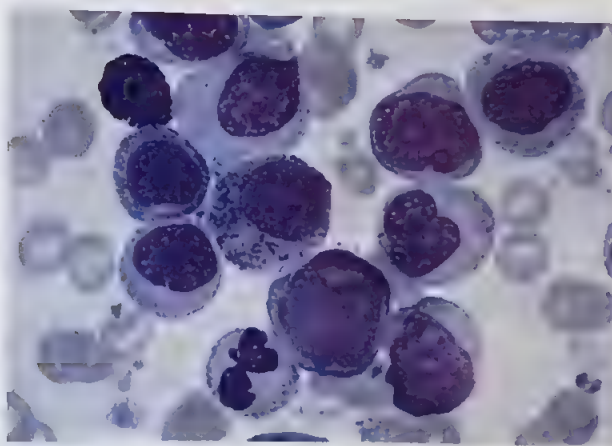


FIGURE 18-7 A bone marrow aspirate from a patient with CML in chronic phase shows granulocytic hyperplasia with orderly maturation through segmented neutrophils. Blasts are not significantly increased. A basophil is at upper left.

to segmented neutrophils is fairly orderly, although the relative increase in myelocytes as seen in the peripheral blood is also present. Blasts constitute less than 10% of the marrow elements in the chronic phase. Basophils and eosinophils are also increased, as in the peripheral blood. Megakaryocytes are also typically increased and clustered in groups of three or more. Gaucher-like histiocytes (histiocytes with blue pigment in the cytoplasm, "sea-blue" histiocytes) can be seen in one-third of patients (Fig. 18-8). As with all MPNs, varying degrees of marrow fibrosis may develop with disease progression (Fig. 18-9).

Leukocyte alkaline phosphatase (LAP), also referred to as neutrophilic alkaline phosphatase, is usually decreased in

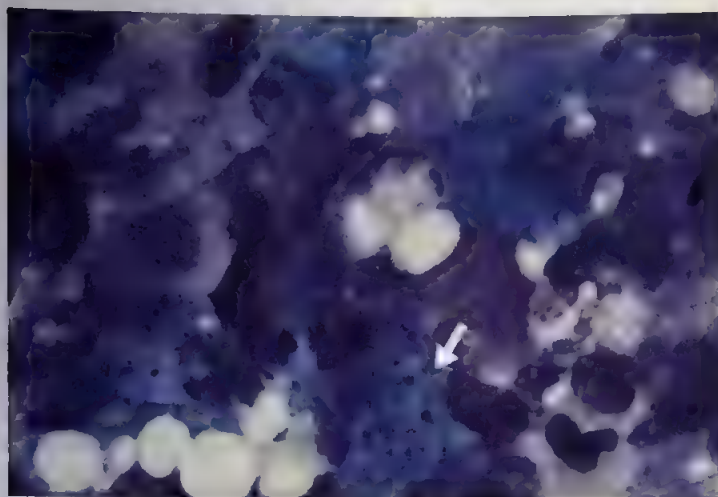


FIGURE 18-8 Pseudo-Gaucher cells (sea-blue histiocytes) in the marrow of a patient with CML.

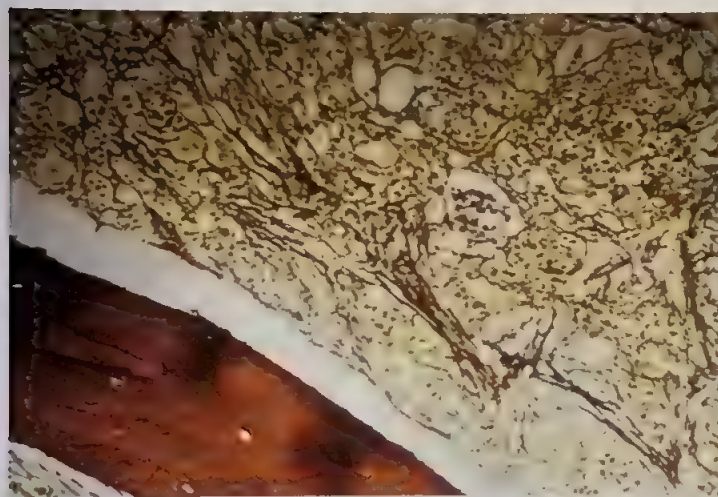


FIGURE 18-9 This bone marrow biopsy from a patient with CML is stained with a reticulin (silver) stain to show reticulin fibrosis, which in this case is quite extensive.

CML. Compare Figure 18-10 showing decreased LAP in a patient with CML with Figure 18-11 showing increased LAP activity in a normal patient with leukemoid reaction. However, LAP may return to normal or may even be increased when a patient with CML becomes infected, during remission of disease with therapy, or when a patient enters blastic phase.

A definitive diagnosis of CML requires the detection of the $t(9;22)(q34.1;q11.2)$ in peripheral blood or *BCR-ABL1* by molecular genetic techniques.³ The $t(9;22)$ (Ph chromosome) can be detected by routine cytogenetics. This technique has the advantage of detecting not only the Ph chromosome but also any other genetic abnormalities that may indicate disease progression. The *BCR-ABL1* gene can be detected by fluorescence in situ hybridization (FISH) or reverse transcriptase polymerase chain reaction (RT-PCR). These have the advantage of specifically detecting the *BCR-ABL1* gene when no structural chromosomal abnormality is seen on routine cytogenetics, as in a patient with an interstitial translocation. The RT-PCR assay is extremely sensitive and is used to detect residual disease or recurrence after treatment. A bone marrow

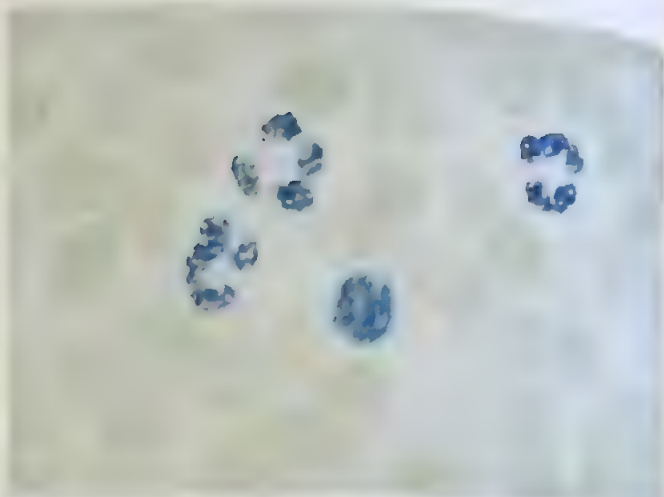


FIGURE 18-10 Leukocyte alkaline phosphatase (LAP) is decreased in peripheral blood neutrophils in this patient with CML. Compare with Figure 18-11.

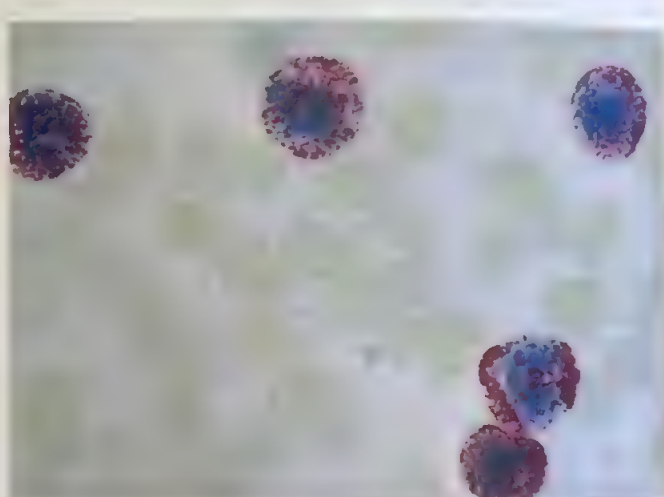


FIGURE 18-11 Leukocyte alkaline phosphatase (LAP) is increased in peripheral blood neutrophils in this patient with a leukemoid reaction related to infection.

aspirate is essential for complete karyotype and morphological examination to confirm phase of disease.³

Most patients are diagnosed while in the chronic phase of CML, and the accelerated phase is suspected when patients become more symptomatic. The blastic phase represents conversion of CML to acute leukemia. The diagnostic criteria for CML by phase are summarized in Table 18-3.¹⁶ Clonal evolution will occur in at least 50% of patients in accelerated phase and in up to 80% of those in blastic phase. This refers to the acquisition of additional cytogenetic abnormalities, such as multiple Philadelphia chromosomes, isochromosome 17, trisomy 8, and other complex karyotypes.³ Mutations or deletions of tumor suppressor genes are also found in the blastic phase and presumably play a role in its development.⁴

ADVANCED CONTENT

To identify the *BCR-ABL1* fusion gene, fluorescence in situ hybridization (FISH) technology is typically utilized.

The FISH assay can be performed in a standard (S) format or a double (D) format. The S-FISH application uses two long, labeled probes that allow detection of straight-forward alterations on chromosome 22q. Alternatively, the D-FISH application is used to identify more complex genetic rearrangements and fusion genes.¹⁷

In patients with an identified *BCR-ABL1* gene fusion, PCR can be used to monitor treatment response with tyrosine kinase inhibitors (TKIs). This analysis can serve as a measurement of minimal residual disease and relapse identification.

Differential Diagnosis

CML must first be distinguished from reactive granulocytic leukocytosis, or leukemoid reaction, which can occur with a variety of stresses including infections and occult malignancies. Both are characterized by neutrophilic leukocytosis with immature forms, but CML will also show basophilia and eosinophilia, as well as the "myelocyte bulge" (presence of more myelocytes than metamyelocytes in peripheral blood).⁹ CML will also show the Ph chromosome and/or *BCR-ABL1* gene, as well as a decreased LAP score. The differential laboratory diagnosis between leukemoid reactions and CML is summarized in Table 18-4.

TABLE 18-4 Differential Diagnosis Between Leukemoid Reaction and Chronic Myelogenous Leukemia

Laboratory Finding	Leukemoid Reaction	CML
Toxic vacuoles	2-4+	0-1+
Toxic granules	2-4+	0-1+
Döhle bodies	Frequent	Rare
Eosinophilia	0	1-3+
Basophilia	0	1-3+
Pseudo-Pelger-Huët	0-1+	Occasional
Karyorrhexis	0-1+	1-2+
Giant bizarre nuclei	0-1+	1-3+
Myelocyte bulge	0	Present
LAP score	High	Low
Ph chromosome	Negative	Positive

LAP = leukocyte alkaline phosphatase

CML may be confused with any of the other myeloproliferative neoplasms (MPNs), especially in its early stages, because all the MPNs are characterized by some degree of neutrophilic leukocytosis and basophilia, while CML can present with increased platelet count and occasionally increased RBC count. The LAP score will be decreased in CML, while it is usually increased in PV. CML diagnosis may be suspected from the CBC and differential results, but most cases are confirmed with *BCR/ABL1* identification.

Prognosis

The prognosis of patients presenting with CML has drastically changed (>80%) with the development of effective tyrosine kinase inhibitor (TKI) therapy.⁴ TKI treatment has significantly reduced the progression of the disorder to the blastic phase, and most patients are successfully treated in the chronic phase. Physicians now consider therapy cessation as the new goal in CML treatment.

Despite the mostly positive responses to TKI therapy, approximately 25% of CML patients show resistance to treatment. Most patients who display therapy resistance prove to have multiple mutations to the *BCR-ABL1* protein. These extra mutations are observed in 25% of cases in the chronic phase and over 70% of blastic phase cases.⁴ Finding an effective form of treatment for these patients is a remaining goal for many researchers.

An increased percentage of blasts directly correlates with a worsening prognosis. Patients with >20% blasts have a worse prognosis than patients with <20% blasts.¹⁹

Several factors may predict blast transformation in CML. These poor prognostic indicators include the presence of karyotypic abnormalities in addition to a single Ph chromosome or *BCR-ABL1* gene, hepatosplenomegaly, thrombocytopenia (less than 100,000/ μ L) or thrombocytosis (more than 500,000/ μ L), extreme leukocytosis (more than 100,000/ μ L),

TABLE 18-3 Diagnostic Criteria for Chronic Myelogenous Leukemia by Phase

Phase of	Diagnostic Criteria
Chronic	<ul style="list-style-type: none"> Increased neutrophils with various degrees of maturation observed Blasts <10% in bone marrow and/or peripheral blood <i>BCR-ABL1</i> mutation demonstrated
Accelerated	<ul style="list-style-type: none"> Blasts 10% to 19% in bone marrow and/or peripheral blood Presence of Ph chromosome or <i>BCR-ABL1</i> mutation with genomic evolution and/or TKI resistance Persistent thrombocytopenia (< 100 \times 10⁹/L) unrelated to therapy, or persistent thrombocytosis (> 1000 \times 10⁹/L) unresponsive to therapy Increasing splenomegaly and increasing WBC count unresponsive to therapy Genomic evolution of multiple genetic abnormalities
Blastic	<ul style="list-style-type: none"> Blasts >20% in bone marrow and/or peripheral blood Presence of Ph chromosome or <i>BCR-ABL1</i> mutation with genomic evolution and/or TKI resistance Genomic evolution of multiple genetic abnormalities Persistent or increasing splenomegaly

and percentage of blast cells in the bone marrow and peripheral blood. The position of the BCR breakpoint has been directly linked with the patient's response to treatment. Genetic complexities increase the probability of a poor response to treatment and a poor prognosis as a result.¹¹

Treatment

The treatment for CML has evolved along with increased understanding and progressive targeting of the underlying molecular pathogenesis. Before the mid-1980s, patients with CML in chronic phase were treated with oral chemotherapy in the form of hydroxyurea or busulfan, intended to reduce leukocytosis, thrombocytosis, and splenomegaly. Although such therapy frequently resulted in hematologic remission, Ph chromosome remained detectable in blood and marrow cells, and the onset of the blastic phase was not delayed. Interferon- α was the first agent to produce cytogenetic as well as hematologic remission in CML. Although complete cytogenetic remissions (absence of Ph chromosome) were rare (13%), those who obtained major cytogenetic remission had significant prolongation of survival as well as a lengthened time to blastic phase.

Imatinib mesylate (Gleevec®), the first TKI to be developed against a specific molecular target, significantly changed the treatment and prognosis for CML. Imatinib directly inhibits the mutant tyrosine kinase activity of the *BCR-ABL1* fusion gene and is now considered the standard frontline treatment for chronic phase CML.⁹ Imatinib has few side effects and has been proven effective at treating older patients (>60 years old).²⁰ In chronic phase CML patients, imatinib produces up to a 98% complete hematologic response rate, with the majority of cases experiencing complete remission.¹⁶ These patients have normal life expectancy with the goal of ending treatment. Even with a good response to TKI treatment, patients are routinely evaluated (every 3 to 6 months) to ensure remission status.

Patients who present in the advanced stages of the disease do not typically show the positive response rate of those in the chronic phase. Additional genetic and cytogenetic alterations result in an increased molecular complexity and reduce the effectiveness of TKI treatments.¹⁸ Patients in the blastic phase have a response rate of <30% and 5-year survival rate is less than 6%.¹³ Patients with Ph + ALL mimic the treatment response of those in blastic phase due to the increased mutations that lead to treatment resistance.¹⁸

Despite the typical good response to imatinib, there are patients who prove to be resistant to treatment. There are now five different TKIs (imatinib, dasatinib, nilotinib, bosutinib, and ponatinib) that have been approved for CML treatment. This variety allows clinicians to tailor treatment options based upon disease phase, risk assessments, response levels, and treatment endpoints.¹⁷

Before imatinib, allogeneic bone marrow transplantation with an HLA-matched related or unrelated donor offered the best treatment for CML. However, due to the high mortality rate, this treatment option is reserved for patients with advanced stage disease or treatment failure/resistance.⁹

ADVANCED CONTENT

Patients who display a resistance to TKI treatment are classified as nonresponders. The resistance is a product of DNA damage associated with impaired DNA repair and the result of various genetic alterations.¹³ The same methodology holds true for patients with BC (blastic phase) CML. The complexity of CML, particularly in the advanced phases, generates much research and advances in treatment options. As stated in the text, imatinib is considered the frontline treatment option for patients with chronic phase CML. The other four TKIs are considered second generation TKIs, and their use targets the more advanced phases of the disorder. Treatment dosages are dependent on the specific patient characteristics, disease progression, life expectancy, and lifestyle. Typically, a combination of TKIs are prescribed. The goal of second generation TKIs is to obtain quick and deeper molecular responses that can address multiple genetic anomalies.

Atypical Chronic Myelogenous Leukemia

Atypical chronic myelogenous leukemia (aCML) is a leukemic disorder that has features of both a myeloproliferative neoplasm and a myelodysplastic syndrome. aCML resembles CML because it is characterized by peripheral blood neutrophilic leukocytosis with immature forms and granulocytic hyperplasia in the bone marrow. However, aCML does not show basophilia, does show significant dysplastic features in the granulocytic line, and always lacks the Ph chromosome and the *BCR-ABL1* gene. Cases of Ph-negative CML that also lack the *BCR-ABL1* gene need to be evaluated molecularly for the presence of *CSF3R*, which has a strong association with CNL (chronic neutrophilic leukemia). The *JAK2/CALR/MPL* mutations are typically not present in patients with aCML.³ Patients with aCML seem to have a poorer prognosis with median survival times of less than 20 months.

Chronic Neutrophilic Leukemia

Chronic neutrophilic leukemia (CNL) is a rare but related chronic myeloproliferative syndrome that is characterized by splenomegaly, peripheral blood neutrophilia, and neutrophilic hyperplasia of the bone marrow.³ The absolute neutrophil count in the peripheral blood is usually greater than 25,000/ μ L, and more than 80% of all white blood cells are neutrophils.³ The neutrophils are present in the mature stages of band and segmented forms, but the percentage of immature myeloid cells (metamyelocytes, myelocytes, promyelocytes, and blasts) is usually low. Basophilia, eosinophilia, and monocytosis are not seen. The bone marrow is hypercellular with predominant granulocytes.³ Unlike CML, CNL does not display a Ph chromosome or the *BCR-ABL1* mutation, and there are no *JAK2/CALR/MPL* mutations. The vast majority of CNL patients do display a presence of *CSF3R* mutation.

Diagnosis requires the identification of the *CSF3R* mutation, or the exclusion of other causes of neutrophilia.²

Chronic Eosinophilic Leukemia, Not Otherwise Specified

Chronic eosinophilic leukemia (CEL), is another related syndrome that is characterized by a clonal expansion of eosinophil precursors with increased peripheral blood eosinophils of greater than 1,500/ μ L.² There is no Ph chromosome, and blasts are less than 2% in the peripheral blood and less than 20% in the bone marrow. The most frequently

found cytogenetic abnormality with CEL is *trisomy 8*. Unlike other eosinophilic neoplasms, CEL does not respond to TKI therapy.²

Myeloproliferative Neoplasms, Unclassifiable

The 2016 evaluation of MPNs by the WHO included the subcategory of myeloproliferative neoplasms, unclassifiable (MPN-U). This subcategory includes neoplasms that cannot be clearly identified as one of the other six disorders listed under the MPN umbrella.³

SUMMARY CHART

- Myeloproliferative neoplasms are a family of acquired clonal disorders of the bone marrow that are characterized by excessive proliferation of one or more nonlymphoid cell lines, with increase in the corresponding cell type in the peripheral blood. All are characterized by splenomegaly.
- The myeloproliferative neoplasms (MPNs) include chronic myelogenous leukemia (CML), polycythemia vera, essential thrombocythemia, primary myelofibrosis, chronic neutrophilic leukemia, chronic eosinophilic leukemia, and MPN-unclassifiable.
- Chronic myelogenous leukemia (CML) may be asymptomatic or symptomatic at presentation. Common symptoms include general malaise; fullness in the upper abdomen related to splenomegaly; symptoms relating to a hypermetabolic state such as night sweats and weight loss; bone tenderness and aching related to bone marrow expansion; and complaints related to anemia. Splenomegaly is a common physical finding.
- CML is characterized by an increased WBC count in peripheral blood, which may be severe. This is related to neutrophilic leukocytosis with immature forms, and basophilia. Anemia is sometimes also present and is usually normocytic. Peripheral blood neutrophils have a low LAP score.
- The bone marrow in CML shows hypercellularity (M:E ratio of 10:1-50:1) related to a marked granulocytic hyperplasia and a moderate megakaryocytic hyperplasia. Pseudo-Gaucher cells and marrow fibrosis may also be present.
- The Philadelphia chromosome (an abnormal chromosome 22) results from a reciprocal translocation between chromosomes 9 and 22, and contains the *BCR-ABL1* fusion gene. The *BCR-ABL1* fusion gene is almost invariably present in CML (98%).
- The *BCR/ABL1* gene enhances tyrosine kinase activity and is the direct cause of the granulocytic proliferation that characterizes CML.
- CML must be distinguished from the other myeloproliferative neoplasms. It must also be distinguished from atypical chronic myelogenous leukemia, which lacks the *BCR-ABL1* fusion gene.
- Imatinib mesylate (Gleevec) currently provides an effective treatment for CML, which can produce long-term remissions.

CASE STUDY 18-1 Chronic Myelogenous Leukemia

REASON FOR VISIT: A 58-year-old white man presented to his physician for evaluation of leukocytosis found incidentally on a complete blood count performed at a health fair. He had no current complaints, but when questioned, he reported feeling of fullness in the left upper quadrant of his abdomen. He reported no recent fevers, night sweats, or weight loss.

PATIENT AND FAMILY MEDICAL HISTORY:

No significant past medical history for the patient or his family. He worked as a supervisor in an automobile assembly plant.

MEDICATION HISTORY: No medications at this time.

PHYSICAL EXAM FINDINGS:

On physical examination, he was found to have splenomegaly, with the spleen tip barely palpable below the costal margin. No hepatomegaly or lymphadenopathy was found.

INITIAL LAB RESULTS:

CBC	
Laboratory Test	Result
CBC	
RBC	$4.04 \times 10^6/\mu\text{L}$
Hb	10.4 g/dL
Hct	30.5%
MCV	91 fL
MCH	28.8 pg
MCHC	34.4%
PLT	$200 \times 10^3/\mu\text{L}$
WBC	$51.20 \times 10^3/\mu\text{L}$
Neutrophils	$36.86 \times 10^3/\mu\text{L}$ (72%)
Lymphocytes	$2.56 \times 10^3/\mu\text{L}$ (5%)
Monocytes	$2.05 \times 10^3/\mu\text{L}$ (4%)
Metamyelocytes	$1.02 \times 10^3/\mu\text{L}$ (2%)
Myelocytes	$3.58 \times 10^3/\mu\text{L}$ (7%)
Promyelocytes	$0.5 \times 10^3/\mu\text{L}$ (1%)
Bone Marrow Evaluation	
M:E ratio	20:1
<i>BCR/ABL1</i>	Positive
Myeloblast	9%

QUESTIONS

1. Identify any abnormalities in the CBC results.
2. What peripheral findings are abnormal?
3. What is the probable patient diagnosis?
4. What is the preferred treatment for this patient?

ANSWERS:

1. WBC count is very elevated and immature neutrophils were found in the peripheral blood, with the majority at the myelocyte stage of maturation. RBC count, hemoglobin, and hematocrit values are low and MCV within normal range, indicating a normocytic anemia.
2. The peripheral smear indicates the presence of immature granulocytic cells, often not present in normal peripheral findings. This should be noted by the laboratory scientist as abnormal.
3. The bone marrow evaluation shows a hyperproliferative marrow with increased myeloid cells. The positive *BCR/ABL1* fusion gene targets a diagnosis of CML and the percentage of myeloblasts identifies the patient is currently in the chronic phase.
4. The preferred treatment for patients with CML in the chronic phase is imatinib (Gleevec), which is a tyrosine kinase inhibitor that targets the effects of *BCR/ABL1* fusion gene.

CASE STUDY 18-2 Chronic Myelogenous Leukemia

REASON FOR VISIT: A 75-year-old white man presented to his physician complaining of fever and fatigue.

PATIENT AND FAMILY MEDICAL HISTORY:

In addition to the fever and fatigue, he reports losing 20 pounds over the last 3 months due to a feeling of fullness. Night sweats began interrupting his sleep, 6 weeks ago.

MEDICATION HISTORY: No medications at this time.

PHYSICAL EXAM FINDINGS:

On physical examination, he was found to have severe hepatosplenomegaly.

INITIAL LAB RESULTS:

CBC	
Laboratory Test	Result
CBC	
RBC	$2.33 \times 10^6/\mu\text{L}$
Hb	10.3 g/dL
Hct	31.1%
MCV	84 fL
MCH	27.3 pg
MCHC	33.1%
PLT	$70 \times 10^3/\mu\text{L}$
WBC	$140.8 \times 10^3/\mu\text{L}$
Neutrophils	$42.2 \times 10^3/\mu\text{L}$ (30%)
Lymphocytes	$7.04 \times 10^3/\mu\text{L}$ (5%)
Monocytes	$1.41 \times 10^3/\mu\text{L}$ (1%)
Metamyelocytes	$25.3 \times 10^3/\mu\text{L}$ (18%)
Myelocytes	$35.2 \times 10^3/\mu\text{L}$ (25%)
Promyelocytes	$4.22 \times 10^3/\mu\text{L}$ (3%)
Myeloblasts	$25.3 \times 10^3/\mu\text{L}$ (18%)
Bone Marrow Evaluation	
M:E ratio	39:1
BCR/ABL1	Positive
Myeloblast	20%

QUESTIONS

1. Identify any abnormalities in the CBC results.
2. Based on the lab results provided, what is the probable diagnosis?
3. Which lab result is most helpful in obtaining a diagnosis?

ANSWERS

1. Severely increased leukocyte count with increased immature neutrophils found in peripheral blood. RBC count, hemoglobin, and hematocrit values are low and MCV within normal range, indicating a normocytic anemia. Thrombocytopenia is also observed.
2. CML is the probable diagnosis based upon the lab results. The patient's blast count is between 10% to 20%, so the accelerated phase is identified.
3. The identification of the *BCR/ABL1* is most helpful in offering a diagnosis. The blast percentage, combined with patient's clinical presentation, allows the identification of accelerated phase.

REVIEW QUESTIONS

1. What is the typical M:E ratio in patients with CML (chronic myelogenous leukemia)?
 - a. 1:10
 - b. 1:5
 - c. 10:1
 - d. 3:1
2. What is the chromosomal abnormality found in CML?
 - a. t(8;14)
 - b. t(9;22)
 - c. t(1;12)
 - d. Trisomy 12
3. Which of the following is consistent with leukemoid reaction?
 - a. Low WBC count
 - b. Basophilia
 - c. Presence of Philadelphia chromosome
 - d. High LAP
4. Which phase of CML carries the worst prognosis and is generally unresponsive to treatment?
 - a. Chronic
 - b. Accelerated
 - c. Blastic
 - d. Refractory
5. Which of the following myeloproliferative disorders is characterized by a decreased LAP score?
 - a. CML
 - b. PMF
 - c. ET
 - d. PV
6. Most patients (95%) are diagnosed in which phase of CML?
 - a. Accelerated phase
 - b. Chronic phase
 - c. Blastic phase
 - d. Fibrotic phase
7. The blastic phase of CML is defined by what percent of blasts found in the peripheral blood or bone marrow?
 - a. 50%
 - b. 10%
 - c. 80%
 - d. 20%
8. At the molecular level, the aberrant conjoining of genetic material from chromosome 9 and chromosome 22 results in a fusion gene called:
 - a. *BCR-ABL1* gene
 - b. *JAK2* gene
 - c. p53
 - d. The blast transformation gene
9. Which of the following is a common risk factor for CML?
 - a. Smoking
 - b. Ionizing radiation
 - c. Family history
 - d. Sulfa drug therapy
10. The Philadelphia chromosome is found in which MPN?
 - a. PV
 - b. CML
 - c. ET
 - d. PMF
11. Characteristics of CML include which of the following?
 - a. Seen only in elderly patients
 - b. Leukopenia
 - c. Splenomegaly
 - d. Enlarged lymph nodes
12. Resistance to treatment is typically found in patients with:
 - a. Low amount of blast cells in peripheral blood
 - b. Multiple genetic alterations
 - c. Basophilia
 - d. Chronic phase CML diagnosis
13. The *BCR-ABL1* mutation is found in which MPN?
 - a. ET
 - b. PV
 - c. CML
 - d. PMF
14. Confirmation of CML and other MPNs requires which analysis?
 - a. LAP Score
 - b. Cytogenetic analysis
 - c. Peripheral blood smear
 - d. Bone marrow analysis
15. Which can be seen in CML?
 - a. Increased LAP score
 - b. The lack of Ph chromosome
 - c. Frequent Dohle bodies
 - d. Basophilia

See answers at the back of this book.

Myeloproliferative Neoplasms II

Polycythemia Vera, Essential Thrombocythemia, and Primary Myelofibrosis

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CHAPTER OUTLINE

Overview of Myeloproliferative Neoplasms

History of the World Health Organization Classification
Genetic Basis of Classical MPNs
General Differentiation of MPNs

Polycythemia Vera

Definition
Incidence
Pathogenesis
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Differential Diagnosis
Treatment

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Definition

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Conclusions

Summary Chart

Case Study 19-1

Case Study 19-2

Case Study 19-3

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 19-1 Describe the origin of myeloproliferative neoplasms.
- 19-2 Correlate myeloid cells with their respective myeloproliferative neoplasms.
- 19-3 Identify the myeloproliferative neoplasms that are negative for Philadelphia chromosome.
- 19-4 Differentiate polycythemia vera from secondary polycythemia and relative erythropoiesis.
- 19-5 Assess clinical complications of polycythemia vera.
- 19-6 Evaluate the diagnostic laboratory findings for polycythemia vera.
- 19-7 Determine the diagnostic criteria for essential thrombocythemia.
- 19-8 Describe the clinical features of essential thrombocythemia.
- 19-9 Assess the key laboratory findings related to a diagnosis of essential thrombocythemia.
- 19-10 Evaluate the clinical findings in primary myelofibrosis.
- 19-11 Determine the laboratory findings for primary myelofibrosis.

The term of **myeloproliferative disorders** was first proposed by William Damashek in a visionary editorial¹ in 1951 to describe disorders characterized by both the excessive proliferation of hematopoietic precursors in the bone marrow and the excessive production of mature blood cells. Despite subsequent discoveries, Damashek's concept of myeloproliferative disorders continues to be universally accepted.

These disorders, by definition, are a group of heterogeneous diseases that originate from the clonal expansion of the hematopoietic pluripotent stem cell resulting in the overproduction of one or more of the formed elements of the blood (i.e., erythrocytes, granulocytes, and platelets in the peripheral blood).² To highlight the clonal nature, the term "disorders" was replaced with "neoplasms" in 2008.³

Overview of Myeloproliferative Neoplasms

Classically, **myeloproliferative neoplasms (MPNs)** have encompassed four clinical entities distinguished by the predominant cell type involved: chronic myelogenous leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). These disorders share molecular and cellular characteristics but differ in phenotype and clinical presentation. The most prominent feature of CML is **Philadelphia chromosome-positive [Ph(+)]** with a 9;22 translocation and *BCR-ABL1* fusion gene present (see Chapter 18). PV, ET, and PMF are [Ph(-)] MPNs that are characterized by increased clonal proliferation of myeloid cells due to *JAK2* mutations. With CML, there is an excessive

production of granulocytes; in PV, it is the overproduction of erythrocytes; and in ET, the overproduction of platelets. PMF is characterized by a prominence of marrow fibrosis and extramedullary hematopoiesis in the liver and spleen. The hallmark physical finding of MPNs is splenomegaly, which occurs in 40% to 99% of patients depending on their specific disorder.⁴ Distinctive features, along with overlapping characteristics of these four disease entities, are presented in Table 19-1. CML was discussed in Chapter 18; this chapter focuses on PV, ET, and PMF.

When anemia is present, it is usually caused by a **myelophthisic anemia** (i.e., bone marrow infiltration of abnormal cell production and/or fibrosis) or increased splenic sequestration due to splenomegaly. The bone marrow is typically hypercellular and varying degrees of fibrosis. The fibrosis is considered to be the bone marrow's response to the increased production of cytokines from genetically altered cells.⁵ As the neoplasm progresses in their severity, fibrosis worsens and hematopoiesis can become apparent in secondary sites of hematopoietic activity, primarily the spleen and liver.⁶

History of the World Health Organization Classification

In 2016, the World Health Organization (WHO) classification of myeloid neoplasms expanded the list of CMPNs to a total of seven entities, adding chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia (CEL), and unclassifiable myeloproliferative diseases. In addition, a category of

myelodysplastic/myeloproliferative diseases was created to include juvenile myelomonocytic leukemia, atypical chronic myeloid leukemia (lacking the 9;22 translocation; [Ph(-)]), and chronic myelomonocytic leukemia.² The term "neoplasm" was introduced in 2008 by the authors of the *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues* to underscore the clonal nature of myeloproliferative disorders and replaced chronic myeloproliferative disorder (CMPD) with "myeloproliferative neoplasms (MPN)."³ The 2008 WHO diagnostic criteria for the traditional *BCR-ABL1*-negative CMPDs (PV, ET, IMF) and the *CEL/HES* CMPDs was also revised. Additionally, the 2016 revision to the WHO classification of myeloid neoplasms includes the following myeloproliferative neoplasms (MPNs): chronic myeloid leukemia (CML) *BCR-ABL1*⁺ (an oncogene or fusion gene); chronic neutrophilic leukemia (CNL); polycythemia vera (PV); primary myelofibrosis (PMF); essential thrombocythemia (ET); chronic eosinophilic leukemia (CEL), not otherwise specified; and MNP, unclassifiable (MPN-U).⁵

Genetic Basis of Classical MPNs

Because of their overlapping features, PV, ET, and PMF have been traditionally grouped into the category of Philadelphia-negative classical MPNs. A key mechanism in these MPNs is an overactive JAK signaling pathway, because of *JAK2* mutations. The identification of a unique base substitution in the *JAK2* gene, the gene encoding Janus kinase 2, in patients with PV, ET, and PMF provided a unifying genetic

TABLE 19-1 Differential Characteristics of the Chronic Myeloproliferative Neoplasms

Characteristics	Chronic Myelogenous Leukemia (CML)	Polycythemia Vera	Essential Thrombocythemia	Primary Myelofibrosis
Hemoglobin	N-s ↓	Marked ↑	N-s ↓	s ↓
WBC count ($\times 10^9/L$)	> 50	12-25	Variable, often mildly ↑	Variable, usually s ↓ early on
Platelet count ($\times 10^9/L$)	Variable-s ↑	450-800	600-2,500	450-1,000, ↓ advanced disease
NRBCs	Common	Rare	Rare	Common
LAP	Mod ↓	Usually s ↑	N	N-mod ↑
Bone marrow	Marked myeloid hyperplasia	Hypercellular, s ↓ iron stores	Hypercellular, marked ↑ megakaryocytes	Fibrosis, dry tap mod-marked ↑
Fibrosis	None-s ↑	None-s ↑	None-mod ↑	mod ↑-marked ↑
Splenomegaly (% patients)	60%-80%	80%	40%-50%	80%-99%
Ph chromosome (<i>BCR/ABL</i> gene fusion)	Positive	Negative	Negative	Negative
<i>JAK2</i> (V617F) mutation	Negative	Positive (65%-97%)	Positive (23%-57%)	Positive (35%-57%)
Special studies	Ph chromosome cytogenetics, FISH, <i>BCR/ABL</i> by PCR	↑ RBC mass determination, ↓ erythropoietin level	Abnormal platelet function tests	Magnetic Resonance Bone Marrow Imaging

WBC = white blood cell; NRBCs = nucleated red blood cells; LAP = leukocyte alkaline phosphatase; FISH = fluorescent in situ hybridization; PCR = polymerase chain reaction; Ph = Philadelphia; JAK = Janus kinase gene.
Reference range: WBC = $5-11 \times 10^9/L$; platelets = $150-450 \times 10^9/L$; N = normal; s ↓ = slight decrease; mod ↓ = moderate decrease; s ↑ = slight increase; mod ↑ = moderate increase; marked ↑ = marked increase.

basis for these disorders.⁷ Identification of somatic mutations of *MPL* (myeloproliferative leukemia virus oncogene) in patients with ET or PMF, of *JAK2* exon 12 mutations in patients with PV, and more recently of somatic mutations of *CALR* (calreticulin gene) in patients with ET and PMF further improved the understanding of the genetic basis of classical MPNs.^{3,8,9}

In general, MPNs are classified as rare cancers, and this chapter focuses on PV, ET, and PMF. Although their WHO classification and novel molecular approach are important to the management of MPN patients, the focus of the chapter remains on a basic approach that is appropriate for medical laboratory scientists. Since PV, ET, and PMF are clonal hematopoietic stem cell (HSC) disorders, understanding HSC biology is critical to MPN diagnosis and management.

General Differentiation of MPNs

Differentiation between MPNs is based upon identified molecular genetic alteration and predominant cell type and morphology in bone marrow and peripheral blood.⁵ The most common MPN genetic mutations already mentioned include *JAK2/CALR/MPL* and *BCR-ABL1* mutations. Three disorders (i.e., PV, ET, and PMF) involve *JAK2/CALR/MPL* mutations, and CML has the diagnostic *BCR-ABL1* mutation.¹⁰ In CML, the bone marrow shows a granulocytic hyperplasia with a dominant increase of **granulocytes** (neutrophils, eosinophils, and basophils) in the peripheral blood.¹¹ (See Chapter 18.) In PV, an erythroid hyperplasia in the bone marrow is accompanied by a corresponding increase of erythrocytes in the peripheral blood, whereas in ET, megakaryocytes are dominantly increased in the marrow and platelets in the peripheral blood. PMF would seem to be the exception because it is characterized by a decrease in most cells (pancytopenia) in the peripheral blood, especially late in its course. However, pre-PMF may show an increase in all cell types, at least before bone marrow fibrosis supervenes to cause pancytopenia.⁵

It should be noted that MPNs can transform from one disorder into another, and all can lead to an acute leukemia, such as acute myeloid leukemia (AML) or acute lymphocytic leukemia (ALL).

ADVANCED CONTENT

The *JAK2/CALR/MPL* mutations are three separate mutations found with MPNs. The most common mutation is the *JAK2* (Janus kinase 2), which is located on chromosome 9q24 and is frequently found in ~95% of PV and 60% of ET and PMF, among other disorders such as RARS-T (refractory anemia with ring sideroblasts associated with marked thrombocytosis).⁶ *CALR* (calreticulin) and *MPL* (myeloproliferative leukemia virus oncogene) mutations are rarely found in PV but are found in ET and PMF.¹² *CALR* mutations are useful for diagnostic clarification in most *JAK2*- or *MPL*-positive patients with ET or PMF. These three mutations work to provide proof of clonality, diagnostic assistance, and prognosis guidance.³ There are rare cases of ET and PMF

where no mutations (called “triple negative”) are found, and diagnosis is based upon other clinical and laboratory characteristics.¹²

The relationship between the two most commonly found genetic alterations in MPNs, *JAK2* and *BCR-ABL1*, is complex and revealing. *BCR-ABL1* directly supports the activation of *JAK2* to promote cell growth and longevity of cells.¹³ Conversely, *JAK2* is responsible for the stability of the *BCR-ABL1* protein. The oncogene *c-MYC* is stimulated in response to *JAK2*, and the overexpression of *c-MYC* plays an essential role in *BCR-ABL1* transformation. These findings provide evidence that the *JAK2* protein controls not only the stability of the *BCR-ABL1* protein but also the oncogenic signaling in *BCR-ABL1* positive cells.^{9,13}

Polycythemia Vera

Polycythemia vera (PV) was first recognized in 1892 by Vaquez and then described by Osler in 1903 as a chronic disease characterized by cyanosis, polycythemia, and splenomegaly.¹⁴ Since then, PV has captured the imagination of physicians in every generation because of its unique clinical features and presentation, and still elusive molecular basis.

Definition

PV is a chronic abnormality of the hematopoietic stem cell characterized by uncontrolled proliferation of erythroid, granulocytic, and megakaryocytic cells, with normal erythropoietin level. PV should be differentiated from both secondary polycythemia and relative erythrocytosis. In secondary polycythemia, only the erythrocytes are increased in number; in relative erythrocytosis, the increase in hematocrit is secondary to a decrease in plasma volume. PV is compared with secondary polycythemia and relative erythrocytosis in Table 19-2.

It is important to distinguish relative from true erythrocytosis and apparent from true polycythemia, as shown in Figure 19-1.¹⁶ In **relative erythrocytosis**, there is an increase in hematocrit values; but in absence of an elevation in red blood cell volume, it is secondary to the decreased plasma volume, as happens in dehydration, diarrhea, vomiting, burn stress-related erythrocytosis, while the erythropoietin level is normal in all listed conditions. Absolute erythrocytosis is a true increase in the number of circulating erythrocytes and hematocrit, as it happens in primary erythrocytosis (PV) and in secondary erythrocytosis, related to high altitude, COPD, and smoking.

In **absolute erythrocytosis**, one can distinguish primary erythrocytosis, as is in PV, with abnormal pluripotential stem cells, excessive proliferation of neoplastic RBCs, WBCs, and platelets, where erythropoiesis predominates, and with a decrease in erythropoietin. On the contrary, in secondary erythrocytosis, there is an excessive proliferation of normal RBCs due to the increase in the erythropoietin level. Secondary erythrocytosis and increase in the erythropoietin levels are happening in the appropriate compensatory response in erythropoietin in response to tissue hypoxia in cardiac and pulmonary diseases, congenital heart failure, high altitude

TABLE 19-2 Features of Polycythemia Vera, Secondary Polycythemia, and Relative Erythrocytosis

Manifestations	Polycythemia Vera	Secondary Polycythemia	Relative Erythrocytosis
Clinical Findings			
Cyanosis	Absent	Present	May be present
Heart or lung disease	Absent	Present	Absent
Splenomegaly	Present in 75%	Absent	Absent
Hepatomegaly	Present in 35%	Absent	Absent
Laboratory Testing and Results			
Red cell mass	Increased	Increased	Normal
Erythropoietin	Decreased (rarely normal)	Increased (rarely normal)	Normal
Arterial O ₂ saturation	Normal	Decreased	Normal
Leukocyte count	Increased in 80%	Normal	Normal
Platelet count	Increased in 50%	Normal	Normal
NRBCs, poikilocytes	Often present	Absent	Absent
LAP	Increased in 70%	Normal	Normal
Bone marrow	Hypercellular; increased erythropoiesis and myelopoiesis; increased megakaryocytes; fibrosis	Increased erythropoiesis	Normal
Serum vitamin B ₁₂	Increased in 75%	Normal	Normal
Culture studies	Autonomous, erythroid proliferation	EPO-dependent colony formation	Not applicable

NRBCs = nucleated red blood cells; EPO = erythropoietin; LAP = leukocyte alkaline phosphatase.

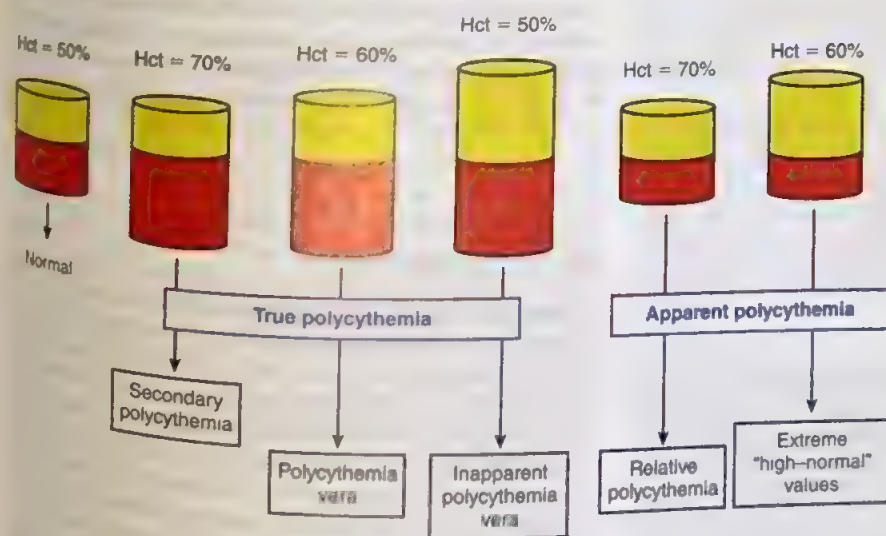


FIGURE 19-1 Erythrocytosis/Polycythemia. The relationship of red blood cell mass and plasma volume in the varieties of polycythemia. Hct = hematocrit. (Adapted from Tefferi A. Polycythemia vera: a comprehensive review and clinical recommendation. *Mayo Clin Proc.* 2003;78(2):174-194.)

defective oxygen transport due to smoking, methemoglobinemia, and hemoglobinopathies. Additionally, inappropriate or pathological secretion of erythropoietin can occur in renal ischemia due to renal tumors, neoplasms such as hepatoma, or some endocrine disorders such as Cushing syndrome (Fig. 19-1).¹⁶ Significant changes were introduced in the 2016 WHO revision of the diagnostic criteria for PV. The updated criteria are listed in Table 19-3, including the bone marrow biopsy as a

major criterion, the dismissal of the erythropoietin-independent erythroid colony test, and, most importantly, the lowering of the hemoglobin threshold to 16.5 g/dL (hematocrit, 49%) and 16.0 g/dL (hematocrit, 48%) for men and women, respectively, compared with 18.5 g/dL and 16.5 g/dL in the 2008 version.¹⁷ Detection of *JAK2* (V617F) mutation in the presence of raised hemoglobin/hematocrit is virtually specific for PV. In case the mutation is absent, analysis for *JAK2*

TABLE 19-3 Revised 2016 WHO Diagnostic Criteria for Polycythemia Vera

Major	<ol style="list-style-type: none"> 1. Hb (>16.5 g/dL in men; >16.0 g/dL in women) or hematocrit (>49% in men; >48% in women) or increased RCM* 2. Bone marrow biopsy[†] showing hypercellularity for age with trilineage growth (panmyelosis) including prominent erythroid, granulocytic, and megakaryocytic proliferation with pleomorphic, mature megakaryocytes (differences in size) 3. Presence of <i>JAK2</i> V617F or <i>JAK2</i> exon 12 mutation
Minor	Subnormal serum erythropoietin level
Criteria Required for Diagnosis	All 3 major or the first 2 major and the minor criterion

CML = chronic myeloid leukemia; ET = essential thrombocythemia; Hb = hemoglobin; LDH = lactate dehydrogenase; MDS = myelodysplastic syndrome; MF = myelofibrosis; PMF = primary myelofibrosis; PV = polycythemia vera; RCM = red cell mass; WHO = World Health Organization.

*More than 25% above mean normal predicted value.

†Bone marrow biopsy may not be required in cases with sustained absolute erythrocytosis defined as hemoglobin levels >18.5 g/dL in men (hematocrit, 55.5%) or >16.5 g/dL in women (hematocrit, 49.5%) if major criterion 3 and the minor criterion are present. However, initial MF can only be detected by performing a bone marrow biopsy; this finding may predict a more rapid progression to overt MF (post-PV MF).

Source: Adapted from Vannucchi AM, Guglielmelli P. What are the current treatment approaches for patients with polycythemia vera and essential thrombocythemia? *Hematology Am Soc Hematol Educ Program*. 2017;2017(1):480-488.

exon 12 mutations should be done in the presence of subnormal serum erythropoietin levels. However, in up to 15% of patients with *JAK2*-mutated PV, the EPO levels may fall within the normal range.¹⁸

The WHO criteria do not mandate bone marrow biopsy for patients with hemoglobin levels >18.5 g/dL (men) and 16.5 g/dL (women) because these levels are invariably equivalent to an expanded red cell mass. However, bone marrow biopsy is recommended to assess bone marrow fibrosis because a grade ≥ 1 fibrosis at diagnosis of PV is associated with a poorer outcome.^{17,19}

Incidence

MPNs are classified as rare cancers because their incidence is lower than 6 per 100,000 persons per year. One study provided the incidence for PV ranging from 0.4 to 2.8 per 100,000 population.²⁰ The median age at diagnosis is 65 years,²¹ with a slight predominance in men.¹⁶ PV is seen in all age groups, including young adults and occasionally even in children. Although rare, there have been reports of familial incidence of PV. The disease also appears to be more common in Jewish individuals of Eastern European descent.

Pathogenesis

The molecular hallmark of PV is a recurrent point mutation (V617F) in *JAK2* that is detected in 95% of PV patients. JAK-STAT (Janus associated kinase-signal transducer and activator of transcription) pathway is one of the critical intracellular signaling in transduction of extracellular signals to

the nucleus to control gene expression.²² Well-regulated JAK signaling is essential for cell production, cell proliferation, and immune function. Intracellular regulators, such as suppressor of cytokine signaling (SOCS), help regulate JAK signaling.²² Cytokines bind to receptors and activate JAKs (*JAK1* and *JAK2*). JAKs activate STATs (signal transducers and activators of transcription), which dimerize and enter the nucleus. Inside the nucleus, the STATs bind to DNA, stimulating the expression of genes related to cell survival, differentiation, and proliferation. A variety of cytokines and growth factors complete their physiological tasks through JAK-STAT pathway, including hematopoiesis, immune regulation, fertility, lactation, growth, and embryogenesis.²²

Factors that affect JAK signaling are *JAK2* mutations, *MPL* mutations, excess cytokines, increased *JAK1* signaling, and impaired negative signaling mechanisms, such as those involving SOCS. So, in the absence of *JAK2* mutations, other mutations can disrupt the normal function of JAK/STAT pathway, resulting in myeloid proliferation. *MPL* mutation is extremely rare in PV. The lymphocyte adaptor protein (*LNK*) is one of a family of adaptor proteins with a critical role in regulation of signaling in hematopoiesis, functioning as a negative regulator of the mutant protein in myeloproliferative neoplasms *JAK2* (V617F). The number of mutations in *LNK* have been described in a variety of myeloproliferative neoplasms, causing increased cellular proliferation with continued erythroid and thrombocytic production.²³

A decade after the description of *JAK2* (V617F) mutation in the MPNs, there is now evidence that MPNs are likely the result of combined genetic dysregulation, with several mutated genes involved in the regulation of epigenetic mechanisms.¹⁸ Epigenetic changes are not due to a change in the DNA sequence; these are reversible modifications that dictate the way in which genes may be expressed (or silenced).

ADVANCED CONTENT

Among the epigenetic mechanisms, DNA methylation is probably the best defined. Currently known MPN-associated mutations now include *JAK2*, *MPL*, *LNK*, *CBL*, *CAI1*, *TET2*, *ASXL1*, *IDH1*, *IDH2*, *IKZF1*, and *EZH2*. Enhancing our knowledge about the mutation profile of patients may allow them to be stratified into risk groups that would aid clinical decision-making.¹⁸ It is possible that the use of epigenetic therapies as alternative pathway targets in combination with JAK inhibitors may be more effective than single agent treatment.

The biological hallmark of PV is the formation of endogenous erythroid colonies with an erythropoietin-independent differentiation. The question that remains is how to relate the endogenous erythroid colony formation and *JAK2* mutation observed in myeloproliferative disorders. *JAK2* activates multiple signal transduction pathways, including activation of several STAT proteins as well as

PI 3-kinase/Akt, Ras/MAPK, and other signaling pathways essential for cytokine-mediated cell survival, proliferation, and differentiation.²⁴ *JAK2* (V617F) stimulates proliferation of erythropoietin-dependent erythroid progenitors, and ectopic expression of *JAK2* (V617F) promotes proliferation of erythropoietin-dependent erythroid progenitors.²⁴ Therefore, it is likely that activation of these downstream pathways caused by the *JAK2* mutation allows for the erythropoietin-independent production of red cells and the formation of endogenous erythroid colony formation as seen in PV.²⁴

Clinical Findings

PV may develop slowly and remain unrecognized for years. Many patients are asymptomatic and are diagnosed incidentally on routine blood work drawn for other evaluations. Patients may present with thrombosis and/or bleeding secondary to erythroid expansion, hyperviscosity, and elevated platelets. Symptoms may also include headache, epistaxis, ischemic or hemorrhagic stroke, angina, myocardial infarction, or claudication (cramping or pain in leg muscles). Patients with underlying atherosclerosis are most likely to experience these symptoms.

Thrombosis is the most common complication in PV and occurs in one-third of the patients.²⁶ Unusual sites of venous thrombus formation including hepatic vein, mesenteric venous, and portal vein thrombosis are common complications of untreated PV. PV patients have, in decreasing order of frequency, headache, weakness, pruritus, dizziness, and sweating. Additionally, pruritus, especially noted after a warm bath, is attributed to the cutaneous mast cell degranulation causing the release of histamine, fibrinolytic factors, or prostaglandins; itching is generalized with the absence of a rash. Erythromelalgia (erythema and painful burning in the hands and feet) is common in PV and is thought to be secondary to platelet thromboxane secretion. Characteristically, these symptoms are ameliorated by the administration of low-dose aspirin. Other complications of PV include gout and an increased risk for peptic ulcer disease. Patients with increased splenomegaly may develop early satiety (fullness) and abdominal pain provoked by splenic infarction.

Splenomegaly is the most common physical finding in PV found in 50% to 80% of the patients. However, in keeping with other myeloproliferative disorders, the manifestations of splenomegaly, myeloid metaplasia, and myelofibrosis are variably expressed at diagnosis and throughout the course of the disease. In the polycythemic phase of the disease, modest hepatomegaly may be observed, as well as plethora. Plethora is a florid, ruddy, cyanotic complexion resulting from an excessive amount of blood; it appears especially on the face but also on the hands, feet, and ears, and is a common finding on physical examination.

Hypertension can occur in 50% of patients, again secondary to vascular congestion. Patients may also have skin excoriations (abrasions), optic fundi vessel engorgement, and gouty

arthritis and tophi. However, 15% to 20% of patients transform to a spent phase with progressive anemia and increasing splenomegaly. The spent phase of PV is associated with increased marrow fibrosis and extramedullary hematopoiesis, primarily in the liver and spleen. The spent phase of PV is often referred to as postpolycythemic myeloid metaplasia and myelofibrosis, and it can be indistinguishable from idiopathic myeloid metaplasia and myelofibrosis, both clinically and by laboratory findings. The spent phase is characterized by constitutional symptoms (fever, night sweats, and weight loss), worsening hepatosplenomegaly secondary to increased extramedullary hematopoiesis, and resultant portal hypertension with ascites, variceal hemorrhage, and portal systemic encephalopathy.

The risk of leukemic transformation in PV at 20 years was found to be approximately 15%.²⁷

Laboratory Testing and Results

Diagnostically, the most significant peripheral blood finding in PV is an absolute erythrocytosis with elevated RBC counts in the range of $6-10 \times 10^{12}/L$, with hemoglobin (Hb) concentrations >18.5 g/dL in males and >16.5 g/dL in females. The hematocrit (Hct) is often higher than 52% (>0.52 L/L) in men and higher than 48% (>0.48 L/L) in women. Because these are measures of concentration, it is important to separate hemoconcentration from a true increase in red cell mass.

Attention must also be paid to the mean corpuscular volume (MCV) because patients with PV occasionally present with iron deficiency secondary to occult, gastrointestinal blood loss, most often resulting from abnormal platelet function. Additionally, repeated therapeutic phlebotomy can also cause iron deficiency. Because of this, a confusing peripheral blood picture can be seen in PV, very similar to thalassemia with significant microcytosis. A normal hematocrit with profound microcytosis is characteristic of PV patients with iron-deficient erythropoiesis secondary to gastrointestinal blood loss, which is frequent in patients with PV (Fig. 19-2).

Assays for serum erythropoietin levels are now readily available and are accurate and reproducible. These assays can be helpful in separating PV from other causes of polycythemia. In PV, erythropoietin levels are characteristically

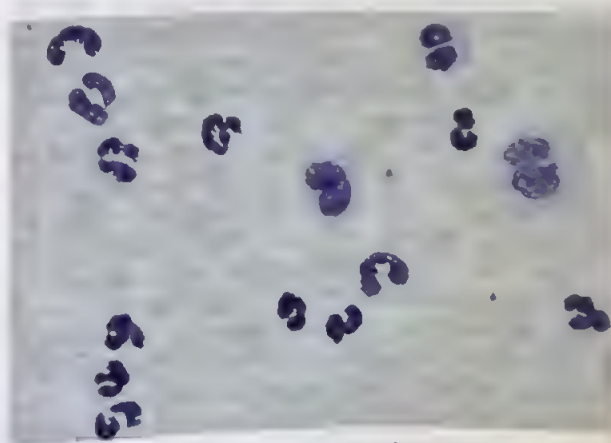


FIGURE 19-2 Peripheral blood smear seen in polycythemia vera. Note hypochromia and increased cellularity (magnification $\times 400$).

low. Peripheral blood assays for erythropoietin-independent endogenous erythroid colony formation growth in vitro can be helpful in confirming the diagnosis^{24,25} but are not readily available.

Characteristically, at presentation there is increased red cell production in intramedullary sites. The erythrocytes are normocytic, normochromic, and have a normal life span. As the disease progresses, extramedullary ineffective hematopoiesis leads to an increasing anisocytosis and poikilocytosis, as well as shortened red cell life span secondary to splenic sequestration. Many patients demonstrate the microcytosis and hypochromia associated with iron deficiency, low serum iron, decreased mean corpuscular volume (MCV), and decreased mean corpuscular hemoglobin concentration (MCHC) occurring in about 50% of patients. This iron deficiency is attributed to a shift of iron into the expanding erythroid mass and to gastrointestinal blood loss, possibly aggravated by platelet dysfunction. The reticulocyte count is usually normal, and only rarely are immature erythrocytes found in the peripheral blood.

Relative and absolute granulocytosis occurs in approximately two-thirds of the patients. The elevation in the total WBC count is usually moderate, with counts in the range of 12 to $25 \times 10^9/L$. However, because only the neutrophils are increased in PV, the total WBC may not accurately reflect disease activity. Occasionally, basophilia and eosinophilia are apparent, and a few metamyelocytes, myelocytes, and even more immature cells may occasionally be seen on examination of the peripheral smear. A shift to the left can be found, and the LAP score is usually higher than 100 (see Table 19-2).

Thrombocytosis is present at the time of diagnosis in about 50% of patients with PV. The platelet count is most often moderately elevated, with counts between 450 and $800 \times 10^9/L$. In approximately 5% of the patients, the platelet count exceeds $1,000 \times 10^9/L$. Platelet life span may be shortened in proportion to the extent of pooling in the spleen. Morphological alterations of platelets include the presence of giant platelets as well as deficient granulation. Most patients with PV form spontaneous megakaryocytic colonies. Platelet aggregation studies may be abnormal but are poorly predictive of the risk of thrombosis or hemorrhage and are not routinely done as part of the evaluation for PV. In most instances, the bleeding time is normal despite in vitro platelet functional abnormalities. Moreover, in general, the bleeding time is a poor predictor of spontaneous or surgical bleeding.

Prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), and fibrinogen levels are generally normal in patients with PV. When performing coagulation tests on patients, the anticoagulant-to-blood ratio must be maintained at a 1:9 ratio. This required ratio can become challenging when the hematocrit is high, as in the case of erythrocytosis, and the plasma volume is decreased relative to the increase in packed red cells. Collecting blood from a patient with erythrocytosis in a normal sodium citrate blood collection tube results in an excess amount of anticoagulant relative to the smaller amount of plasma. Therefore, appropriate collection requires a reduction in the amount

of sodium citrate anticoagulant to obtain the required 1:9 anticoagulant-to-blood ratio.

The RBCs in the peripheral smear are generally normocytic, normochromic. Occasionally, basophilia and immature granulocytes may be seen but not circulating blasts. Giant platelets can occasionally be found. The bone marrow characteristically shows panmyelopathy with erythroid, myeloid, and megakaryocytic hyperplasia, in contrast to secondary erythrocytosis, in which only erythroid hyperplasia is evident (Fig. 19-3). Megakaryocytes are often increased in number and are atypical, with deeply lobated nuclei. They are often arranged around marrow sinusoids or in a paratrabecular location. Bone marrow iron stores, demonstrated by Prussian blue staining, are reduced or absent. This reduction results from the shift of iron to the expanded red cell mass. Stainable iron may be entirely absent in patients with chronic blood loss or after multiple phlebotomies. Fibrosis is rare (less than 5%) early in the course of the disease but may increase to 20% after 10 to 15 years and can reach up to 50% after 20 years with progression to the spent phase. Serial biopsies performed over a period of many years showed progressive increase in reticulin deposits even before the spent phase develops. As the disease runs its course, cellularity usually decreases, although megakaryocytosis may persist.

The leukocyte alkaline phosphatase (LAP) score is elevated in PV in contrast to CML in which the LAP score is decreased (Fig. 19-4). Uric acid, LDH, and vitamin B₁₂ levels can be elevated as well, secondary to high cellular turnover. Hyperuricemia and uricosuria are found in 40% of patients with PV at the time of presentation, reflecting the increased synthesis and degradation of cellular nucleotides, and can be seen in other hyperproliferative marrow disorders. Most patients with elevated uric acid levels remain asymptomatic, but uncommonly, clinical gout may develop. A low to normal erythrocyte sedimentation rate (ESR) is commonly present in PV patients. The increased hematocrit, as well as the elevated ratio of red cell membrane to plasma fibrinogen and globulins, may account for this finding.

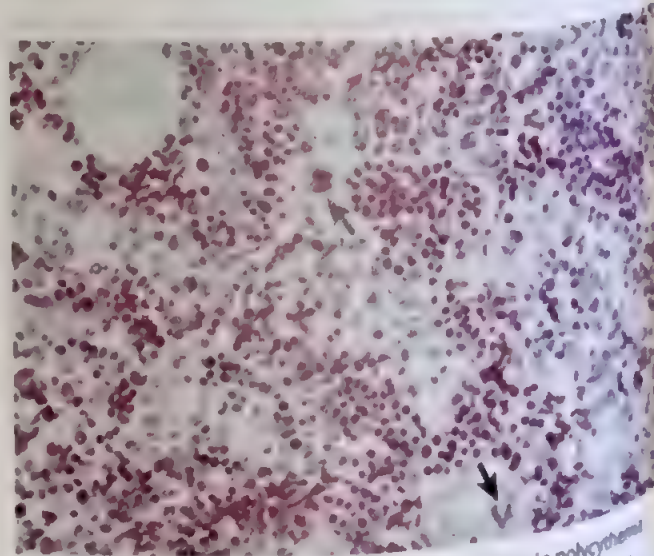


FIGURE 19-3 Bone marrow showing panhyperplasia in polycythemia vera. Note increased number of megakaryocytes (arrows). H&E stain (low power).

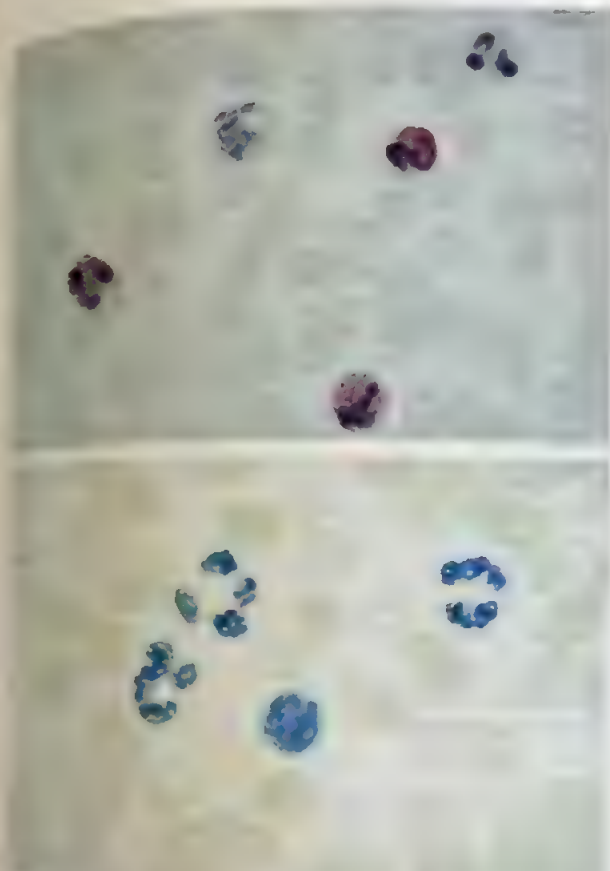


FIGURE 19-4 Leukocyte alkaline phosphatase (LAP) stain of peripheral blood showing increased activity in polycythemia vera (red staining). LAP negative stain in CML (bottom).

Peripheral blood assays for erythropoietin-independent endogenous erythroid colony formation growth in vitro, as discussed before,^{24,25} can be helpful in confirming the diagnosis but are not readily available.

Hematopoietic disorders are often driven by genetic mutations and epigenetic alterations, as new advanced molecular techniques are utilized in hematology.²⁸ Because of the high frequency of *JAK2* (V617F) mutation in PV, peripheral blood screening for *JAK2* (V617F) is recommended as a first step in the initial evaluation of patients suspected of having PV. Since greater than 95% of patients with PV exhibit the *JAK2* mutation (V617F), in its absence, the diagnosis of PV is unsupported.

ADVANCED CONTENT

Cytogenetic abnormalities, such as chromosomal aneuploidy and partial deletions, can be found. Trisomy 8 or 9, an abnormally long chromosome 1, and partial deletions of chromosomes 13 and 20 are the most consistent chromosomal abnormalities. Abnormal karyotype that adversely affects overall and transformation-free survival²⁹ was reported in approximately 20% of patients (+ 9, + 8, and

20q- being the most frequent). Overall, the presence of cytogenetic and chromosomal abnormalities increases after years of treatment and may have an effect on the risk of transformation and progression to acute leukemia.²⁹

Differential Diagnosis

It is essential that PV be differentiated from the more benign causes of secondary erythrocytosis and relative polycythemia so that the effective therapy may be initiated. However, the differential diagnosis between primary (PV) and secondary polycythemia may not always be straightforward.

The arterial oxygen saturation should be >92% as indicated in the PV diagnostic criteria. Patients with arterial oxygen saturations below 92% should be suspected of having secondary polycythemia related to hypoxia. Secondary polycythemia may be appropriate (secondary to hypoxia from chronic obstructive pulmonary disease [COPD], right to left cardiac shunts, and hemoglobin with increased oxygen affinity) or inappropriate as with erythropoietin secretion secondary to renal cysts, or hypernephroma. Please refer to Table 19-2 and Figure 19-1 for features that differentiate PV from secondary polycythemia and relative erythrocytosis. A brief list of the causes of secondary polycythemia is given, but its management is beyond the scope of this chapter. Unlike secondary polycythemia, PV is a panmyelopathy and is usually associated with thrombocytosis and leukocytosis.

Treatment

Generally, the purpose of treatment is to provide relief of symptoms, minimize long-term complications, such as thrombosis and hemorrhage, and to avoid increasing the risk of leukemic transformation. The most common treatment modality utilized in PV is therapeutic phlebotomy. Reduction of blood volume (usually one unit of whole blood, 450 cc) can be performed weekly or even twice weekly in younger patients to control symptoms. The Hct target range is less than 45% (0.45 L/L) for men and less than 42% (0.42 L/L) for women. Therapeutic phlebotomy decreases the erythrocyte count while reducing blood viscosity and alleviating symptoms. However, these blood collections can result in iron deficiency and therefore may be required less frequently, at every 3 to 6 months. Aspirin therapy (81-100 mg once daily) plus therapeutic phlebotomy with a target hematocrit of 45% (0.45 L/L) in all male and female patients with PV, regardless of risk status, has been recommended.

Hydroxyurea, a traditional cytoreductive agent used in PV, can reduce the thrombosis rate, normalize the platelet count and spleen size, and ameliorate hypercatabolic symptoms. Subcutaneous injections of interferon-alpha can also control blood counts, splenomegaly, and hypercatabolic symptoms. However, side effects such as fever, fatigue, depression, anorexia, nausea, and vomiting can limit that treatment's usefulness. Anagrelide, an oral imidazoquinazoline derivative that decreases platelet production and inhibits platelet aggregation, has also been used in PV, primarily for the control of thrombocytosis.

Cytoreductive therapy may be reserved for high-risk patients who are older than 60 years, have thrombotic risk factors, prior thrombotic events, and/or increased platelet counts. In addition, *JAK2* inhibitor therapy^{15,17,30} and epigenetic therapies¹⁸ are reasonable to be considered for high-risk patients. *JAK2* inhibitor therapy, in the presence of protracted pruritus or markedly enlarged splenomegaly, has been shown to be refractory to the aforementioned drugs.³¹ While hydroxyurea remains the first-line treatment for cytoreduction in most “high-risk” ET and PV patients, the disease-modifying potential of interferon (IFN) is promising and could make it a preferred option for selected patients.³² Advances in molecular testing will enable a more individualized approach to prognosis and treatment selection.³²

A cytoreductive therapy in “high-risk” patients is recommended with the first-, second-, and third-line drugs of choice being hydroxyurea, pegylated interferon- α , and busulfan, respectively.³¹

CRITICAL THINKING QUESTION

19-1 If there are increased numbers of RBCs in PV, why is the ESR normal or even low in these patients?

See answers to all Critical Thinking Questions at the back of this book.

Essential Thrombocythemia

Initially, essential thrombocythemia was referred to as hemorrhagic thrombocythemia and identified as thrombocytosis that was secondary to other disorders. Considerable controversies existed regarding essential thrombocythemia, and it was not until 1960 that essential thrombocythemia was established as a separate disease entity on a clinicopathologic basis.³³

Definition

Essential thrombocythemia (ET) is a rare but serious myeloproliferative neoplasm (MPN) characterized by marked thrombocytosis with bone marrow megakaryocytic hyperplasia and a tendency to develop thrombotic and hemorrhagic complications.^{7,17,20,26,34} The WHO revised diagnostic criteria for ET in 2016 are given in Table 19-4. There was no substantial change in the diagnostic criteria of ET compared with the previous version (2008), except for including *CALR* mutations among the major criteria. These guidelines include:

1. Platelet count $\geq 450 \times 10^9/L$.
2. Bone marrow biopsy showing proliferation mainly of the megakaryocyte lineage with increased numbers of enlarged, mature megakaryocytes with hyperlobulated nuclei. No significant increase or left shift in neutrophil granulopoiesis or erythropoiesis and very rarely minor (grade 1) increase in reticulin fibers.
3. Not meeting WHO criteria for *BCR-ABL1* CML, PV, PMF, MDSs, or other myeloid neoplasms.
4. Presence of *JAK2*, *CALR*, or *MPL* mutation.

Incidence

One study provided an incidence of ET from 0.38 to 1.7 per 100,000 persons per year.²⁰ It seems to be the most frequent

TABLE 19-4 Revised 2016 WHO Diagnostic Criteria for Essential Thrombocythemia

Major	<ol style="list-style-type: none"> 1. Platelet count $\geq 450 \times 10^9/L$ 2. Bone marrow biopsy showing proliferation mainly of the megakaryocyte lineage with increased numbers of enlarged, mature megakaryocytes with hyperlobulated nuclei. No significant increase or left shift in neutrophil granulopoiesis or erythropoiesis and very rarely minor (grade 1) increase in reticulin fibers. 3. Not meeting WHO criteria for <i>BCR-ABL1</i> CML, PV, PMF, MDSs, or other myeloid neoplasms 4. Presence of <i>JAK2</i>, <i>CALR</i>, or <i>MPL</i> mutation
Minor	Presence of a clonal marker or absence of evidence for reactive thrombocytosis
Criteria Required for Diagnosis	All 4 major criteria or the first 3 major and the minor criterion

CML = chronic myeloid leukemia; ET = essential thrombocythemia; Hb = hemoglobin; LDH = lactate dehydrogenase; MDS = myelodysplastic syndrome; MF = myelofibrosis; PMF = primary myelofibrosis; PV = polycythemia vera; RCM = red cell mass; WHO = World Health Organization.

Source: Adapted from Vannucchi AM, Guglielmelli P. What are the current treatment approaches for patients with polycythemia vera and essential thrombocythemia? Hematology Am Soc Hematol Educ Program. 2017;2017(1):480-488.

of the MPNs, with approximately 6,000 people diagnosed annually in the United States. The median age at diagnosis is 68 years,¹⁹ although as many as 20% of patients may be younger than 40 years of age. The disease is more common in women, with a female-to-male ratio of 1.6. Interestingly, the increased incidence of ET in women is most apparent in women between 30 and 50 years of age. Although MPN patients overall have reduced life expectancy compared with the general population, the relative survival rate is lower in PMF compared with PV, and in PV compared with ET.³⁵ Interestingly, survival of patients with WHO-defined ET is similar to that of the sex- and age-standardized European population. Excess of mortality in MPN patients is attributable to death from hematologic malignancies or from bacterial infections, and in young patients also from cardiovascular and cerebrovascular disease. However, overall there has been a clear improvement in survival between 1973 and 2005, mainly because of decreased probabilities of dying as a result of these complications.³⁵

Pathogenesis

As in myelofibrosis and polycythemia vera, dysregulated signaling in the JAK pathway plays a role in the pathophysiology of ET. The *JAK2* (V617F) mutation occurs in about 55% of patients with ET,²² and a mutation in the thrombopoietin receptor gene *MPL* occurs in about 4% of affected patients.

ADVANCED CONTENT

In PV, *JAK2* (V617F) mutation also results in increased responsiveness to erythropoietin (EPO). Serum thrombopoietin (TPO), the major megakaryocytic growth and

Enumeration of platelets in whole blood counters can be problematic due to variations in platelet size and spontaneous platelet aggregation. Therefore, review of peripheral smears for the presence of platelet aggregates is mandatory to avoid errors in platelet counting.

A mild normocytic, normochromic anemia may be present in up to 50% of patients, although the hemoglobin value is not usually less than 10 g/dL. Recurrent hemorrhage due to low platelet count typically leads to iron deficiency anemia, which corresponds with a microcytic, hypochromic blood picture. Erythrocyte morphological findings reflect hyposplenism or autosplenectomy that may occur with splenic infarction and atrophy. These findings include the presence of Howell-Jolly bodies, nucleated erythrocytes, Pappenheimer bodies (siderotic granules), target cells, acanthocytes, and poikilocytosis.

For medical laboratory scientists, it is important to note that thrombocytosis with variation in size and shape and platelet aggregates and clusters could interfere with RBC counts on automated cell counters and falsely elevate these counts. Therefore, the hemoglobin determination could be a better parameter to assess anemia.

A leukocytosis can be present in about one-third of patients. Neutrophilia is observed in the majority of patients with elevated WBC counts. The LAP score is variable but most commonly is normal.

The bone marrow in patients with ET exhibits marked hyperplasia with a striking increase in the megakaryocytic component. Megakaryocytes are typically larger than normal and may be dysplastic in appearance with abundant cytoplasm and hyperlobulated nuclei, usually occurring in loose clusters or dispersed in the marrow (Fig. 19-6). Significant marrow fibrosis is generally not seen. Stainable iron is normal to slightly decreased in most cases, and increased reticulin content is often seen.

Platelet function studies reveal a variety of abnormalities in some patients. Abnormal platelet aggregation to epinephrine, collagen, adenosine diphosphate (ADP), and ristocetin are quite frequent. Studies have demonstrated both normal

bleeding times (even in the case of patients with hemorrhagic tendencies) and prolonged bleeding times. PT and PTT are usually normal. Reduced platelet factor 3 (PF3), reduced platelet adhesion, low protein S levels, and nucleotide storage pool defects have all been reported in association with ET. These abnormalities are not useful in predicting thrombosis or hemorrhage. In addition, the severity of thrombocytosis does not necessarily correlate with the presence, absence, or severity of symptoms. Falsely elevated serum potassium and phosphorus levels will sometimes be seen as result of *in vitro* release of potassium from platelets. Increase in serum uric acid, lactate dehydrogenase, and acid phosphatase can be caused by the increase in a cellular turnover.

Approximately 50% to 60% of ET patients have a mutation of the *JAK2* gene; 30% *CALR* gene mutation; 3% *MPL* gene mutation; and about 12% of patients with ET are triple-negative for these gene mutations.^{17,36} The finding of the *JAK2* mutation supports the existence of more than one type of etiology of this disease. Red cell mass determinations and erythropoietin levels may be necessary in patients with borderline hematocrits to rule out PV. Marrow karyotype is generally normal. Clinical diagnosis in patients with ET can be aided by mutational findings of *JAK2*, *CALR*, or *MPL* genes. However, although there is a diagnostic value of mutations in the three genes *JAK2*, *CALR*, and *MPL*, the effect of the mutation type correlated to the clinical outcome for patients has not been fully elucidated.³⁶

Differential Diagnosis

Essential thrombocytosis must be differentiated from a secondary reactive thrombocytosis. This differential diagnosis can be difficult, and ET often remains a diagnosis of exclusion. Essential thrombocytosis or thrombocythemia must be differentiated from the various causes of reactive thrombocytosis listed in Table 19-5. The features differentiating ET from reactive thrombocytosis are summarized in Box 19-1.

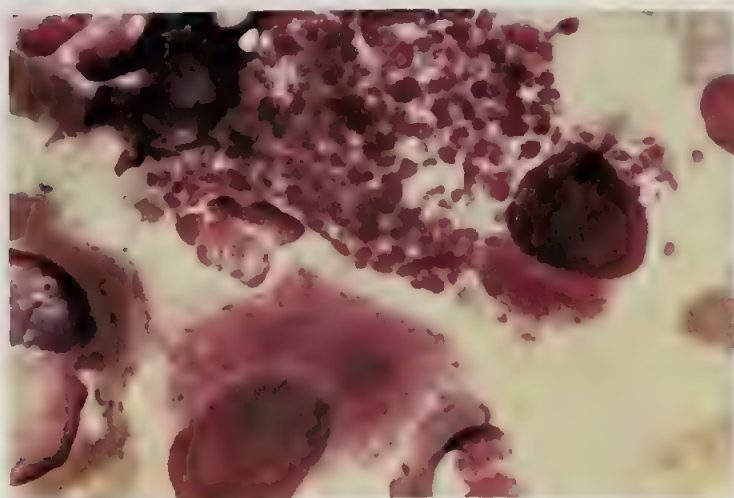


FIGURE 19-6 Essential thrombocythemia, bone marrow. Note increased megakaryocytes. (From Hyun BH. Morphology of Blood and Bone Marrow. Philadelphia: American Society of Clinical Pathologists, Workshop 5121; 1983, with permission.)

TABLE 19-5 Differentiating Laboratory Results Between Essential Thrombocytosis and Reactive Thrombocytosis

Laboratory Results	Essential Thrombocytosis	Reactive Thrombocytosis
Platelet count >1,000,000	Frequent	Infrequent
Leukocytosis	Frequent	Can be present in reactive states
Anemia	Normocytic, normochromic	Microcytic, hypochromic (secondary to iron deficiency)
Splenomegaly	<50%	Not present
Clustering megakaryocytes in bone marrow	Common	Not present
Bone marrow fibrosis	20%	Not present

BOX 19-1 Causes of Reactive Thrombocytosis

- Acute hemorrhage
- Splenectomy and hyposplenism
- Postoperative
- Malignancy
- Chronic inflammatory disorders
- Chronic infection
- Iron deficiency anemia
- Drug-induced
- Trauma
- Hemolytic anemia
- Myelodysplastic diseases
- Graft-versus host disease
- Vitamin E deficiency
- Hyperadrenalism
- Rebound recovery from thrombocytopenia
- Exercise

Thrombocytosis is often present in patients with a wide variety of inflammatory, malignant, infectious, and trauma-associated conditions. Reactive thrombocytosis, even when present for weeks or months, is usually well tolerated in these patients and is usually not associated with thrombosis or hemorrhage. Bone marrow examination may not allow a distinction between ET and reactive thrombocytosis and should be avoided when alternative etiologies are readily apparent. The platelet count in secondary (reactive) thrombocytosis seldom exceeds $1,000 \times 10^9/L$, and commonly falls in the range of 500 to $750 \times 10^9/L$; the platelet morphology and function are generally normal. Other markers of the acute phase response such as interleukin-6 (IL-6), plasma fibrinogen, and C-reactive protein are elevated in the acute phase response to infection, trauma, and inflammation, and may help in differentiating reactive thrombocytosis from ET. Some patients with ET demonstrate erythropoietin-independent endogenous erythroid colony formation, which is never found in reactive thrombocytosis. When endogenous erythroid colony formation is found, the presence of an MPN such as ET is supported.

When the platelet count is persistently greater than $600 \times 10^9/L$ and the bone marrow demonstrates predominant megakaryocytic hyperplasia, the diagnosis of ET should be investigated, and differentiation from other MPNs with thrombocytosis is necessary. Because there are no unique clinical, hematologic, or histopathologic findings in this disease, it is by nature a diagnosis of exclusion. Molecular analysis of the bone marrow should be performed to ensure the absence of *BCR-ABL1* gene rearrangements. (See Chapter 18 on Chronic Myelogenous Leukemia.)

Early PMF is often associated with extreme thrombocytosis. However, there is usually marked splenomegaly, a leukoerythroblastic blood picture, teardrop poikilocytosis, and the characteristic myelofibrotic involvement of the bone marrow (increased reticulin and collagen fibrosis). In ET, less than one-third of the biopsy area demonstrates fibrosis.

Myelodysplastic syndromes associated with thrombocytosis are usually present with a more severe degree of anemia that

is often macrocytic in appearance compared with that seen in patients with ET. In addition, the presence of either cytogenetic abnormalities (5q⁻, deletion 7, trisomy 8) or ringed sideroblasts in the bone marrow denotes a myelodysplastic syndrome as the cause of the associated thrombocytosis rather than essential thrombocythemia. (See Chapter 20 on Myelodysplastic Disorders.)

CRITICAL THINKING QUESTION

19-2 Due to the increased likelihood of platelet aggregates and giant platelets in patients with ET, how might you expect the automated CBC results to be affected?

Treatment

The therapeutic approach to ET depends on many factors, including the patient's age and childbearing potential, the height of the platelet count, and, most importantly, the presence and duration of symptoms.

ADVANCED CONTENT

Cytoreductive therapy includes use of hydroxyurea, anagrelide, and ruxolitinib. Current drug therapy for essential thrombocythemia (ET) and polycythemia vera (PV) is neither curative nor has it been shown to prolong survival. Fortunately, prognosis in ET and PV is relatively good, with median survivals in younger patients estimated at 33 and 24 years, respectively.³¹ Therefore, when it comes to treatment in ET or PV, it is better to avoid exposing patients to new drugs that have not been shown to be disease-modifying and whose long-term consequences are suspect (e.g., ruxolitinib). Furthermore, the main indication for treatment in ET and PV is to prevent thrombosis, and, in that regard, none of the newer drugs have been shown to be superior to the time-tested older drugs (e.g., hydroxyurea).³¹ Currently, three major factors for thrombosis must be considered—history of thrombosis, *JAK2/MPL* mutations, and advanced age—to group ET patients into four risk categories: “very low risk” (absence of all three risk factors), “low risk” (presence of *JAK2/MPL* mutations), “intermediate-risk” (presence of advanced age), and “high-risk” (presence of thrombosis history or presence of both *JAK2/MPL* mutations and advanced age). Tefferi et al. provided a treatment algorithm that is risk-adapted and based on evidence and decades of experience.³¹

The most important first step in the management of ET is to confirm the accuracy of the diagnosis and make sure that other myeloid neoplasms, which might mimic ET in their presentation (e.g., prefibrotic PMF, masked polycythemia vera, chronic myeloid leukemia, refractory anemia with ring sideroblasts, and thrombocytosis), are excluded.³¹ Plateletpheresis

is used to reduce platelet counts, and hydroxyurea is used to suppress megakaryocyte production in the bone marrow. Anagrelide has been evaluated for its efficacy and safety as a first-line therapy for ET. However, these studies suggested that it was not better than hydroxyurea and might be harmful for patients who may experience a higher incidence of arterial thrombosis, bleeding complications, and fibrotic progression.³¹

Additionally, therapeutic trials with interferon show improvement in both clinical symptoms and hematological parameters; however, withdrawal of interferon, leads to recurrence of thrombocytosis. In conclusion, while hydroxyurea remains the first-line treatment for cytoreduction in most ET and PV patients, the disease-modifying potential of interferon is promising and could make it a preferred option for selected patients. The advances in molecular testing will definitively enable a more individualized approach to prognosis and the treatment selection.³²

Primary Myelofibrosis

Primary myelofibrosis (PMF), also called idiopathic myelofibrosis or idiopathic myeloid metaplasia with myelofibrosis, was first introduced by Heuck in 1879 when he described a case of a 24-year-old butcher who had severe fatigue for 1 year. On examination, he showed severe anemia, leukocytosis with myeloid immaturity, and marked hepatosplenomegaly. A severe osteosclerosis and extramedullary hematopoiesis was shown on autopsy, indicating that myelofibrosis with myeloid metaplasia in this case was distinct from leukemia. PMF was formally included as one of the myeloproliferative disorders in 1951.

Definition

Primary myelofibrosis (PMF) is a clonal hematopoietic stem cell disorder characterized by bone marrow fibrosis, symptom burden, splenomegaly, and cytopenias. PMF is a disease with significant heterogeneity and symptom burden. Patients with PMF show the whole range of presentations starting from those who are essentially asymptomatic to those who have severe symptoms that affect their quality of life and daily functioning.³⁸ PMF is a progressive hematopoietic stem cell disorder characterized by bone marrow and blood cell abnormalities. Bone marrow fibrosis and insufficient hematopoiesis drive extramedullary hematopoiesis, particularly in the spleen. Therefore, PMF is characterized by fibrosis of the marrow, extramedullary hematopoiesis, or myeloid metaplasia of the spleen and liver. This triad gives rise to moderate to marked splenomegaly and hepatomegaly, leukoerythroblastosis, and teardrop poikilocytosis of the peripheral blood.

PMF is known by at least 20 synonyms. Some of the terms that have been used most frequently are agnogenic myeloid metaplasia (AMM), myelosclerosis, osteosclerosis, aleukemic myelosis, and chronic idiopathic myelofibrosis (CIMF) or primary myelofibrosis (PMF). The term myelofibrosis with myeloid metaplasia, or just idiopathic myelofibrosis (IMF), highlights the essential features of fibrosis and extramedullary hematopoiesis.

PMF can be divided into two stages: a prefibrotic/early stage and a fibrotic stage. The prefibrotic stage is characterized by a granulocytic and megakaryocytic myeloproliferation, decreased number of erythroid precursors, lack of fibrosis, and abnormalities in megakaryopoiesis characterized by atypical megakaryocytes. Clinically, this early stage of PMF mimics the early stages of ET and PV. The fibrotic stage is characterized by fibrosis of the marrow, extramedullary hematopoiesis (splenomegaly), and leukoerythroblastosis of the peripheral blood.

The WHO updated and revised diagnostic criteria for early PMF in 2016 are given in Table 19–6. Compared with the previous 2008 WHO version, a remarkable action was the identification of a prefibrotic/early form of PMF as a distinct entity with respect to ET and overt fibrotic PMF. Increased numbers of large and mature-appearing megakaryocytes with hyperlobulated nuclei, in the context of normal, age-adjusted bone marrow cellularity, are typical of ET. In pre-PMF, megakaryocytes display abnormal maturation with hyperchromatic and irregularly folded nuclei, form clusters, and are surrounded by increased cellularity with granulocytic proliferation and often decreased erythropoiesis.¹⁷ Other minor criteria may also help in the differential diagnosis between ET and pre-PMF.

TABLE 19-6 Revised 2016 WHO Diagnostic Criteria for Prefibrotic PMF	
Major	1. Megakaryocytic proliferation and atypia, without reticulin fibrosis >grade 1, accompanied by increased age-adjusted BM cellularity, granulocytic proliferation, and often decreased erythropoiesis 2. Not meeting the WHO criteria for BCR-ABL + CML, PV, ET, MDS, or other myeloid neoplasms 3. Presence of JAK2, CALR, or MPL mutation or in the absence of these mutations, presence of another clonal marker, [†] or absence of minor reactive bone marrow reticulin fibrosis ^{§§}
Minor	a. Anemia not attributed to a comorbid condition b. Leukocytosis ≥11 × 10 ⁹ /L c. Palpable splenomegaly d. LDH increased to above upper normal range
Criteria Required for Diagnosis	All 3 major criteria, and at least 1 minor criterion

CML = chronic myeloid leukemia; ET = essential thrombocythemia; Hb = hemoglobin; LDH = lactate dehydrogenase; MDS = myelodysplastic syndrome; MF = myelofibrosis; PMF = primary myelofibrosis; PV = polycythemia vera; RCM = red cell mass; WHO = World Health Organization.
[†]In the absence of any of the 3 driver mutations, the search for the most frequent accompanying mutations are of help in determining the clonal nature of the disease.
^{§§}Minor (grade 1) reticulin fibrosis secondary to infection, autoimmune disorder or other chronic inflammatory conditions, hairy cell leukemia or other lymphoid neoplasm, metastatic malignancy, or toxic (chronic) myelopathies.
Source: Adapted from Vannucchi AM, Guglielmelli P. What are the current treatment approaches for patients with polycythemia vera and essential thrombocythemia? Hematology Am Soc Hematol Educ Program. 2017;2017(1):480-488.

The prognostic relevance of distinguishing ET from pre-PMF is supported by several studies in terms of median survival, with ET having a better prognosis.³⁹

Incidence

Recall that MPNs are classified as rare cancers because their incidence is lower than 6 per 100,000 persons per year. One study provided the following incidence estimates: PV from 0.4 to 2.8, ET from 0.38 to 1.7, and PMF from 0.1 to 1.0 per 100,000 persons per year.²⁰ These disorders generally occur in middle- or advanced-age adults, with a median age of 65 years for PV, 68 years for ET, and 70 years for PMF.²¹ In the United States, PMF has an incidence of 1.5 per 100,000 per year, occurring mainly in middle-aged and elderly patients with a median age of 70 years at presentation.

Pathogenesis

Although rare in childhood, familial occurrences have been reported in several generations within families in the absence of any causative environmental agent. It seems to be less common in individuals of African or Spanish descent. However, PMF has also been linked to exposure to some chemicals and ionizing radiation, and high incidence of PMF has been reported in Hiroshima survivors and noted in patients given thorium-based radiographic contrast material. Myelofibrosis has also occurred secondary to chronic infections, especially tuberculosis, histoplasmosis, and even after myocardial infarction.

The exact cause of PMF is unknown. Some evidence has indicated that PMF originates at the level of the CD34⁺ hematopoietic stem cell. A defective stem cell "niche" within the bone marrow has been postulated for PMF.⁴⁰ However, studies did not find any significant relationship between the clinicopathologic and laboratory characteristics and peripheral and bone marrow CD34⁺ from bone marrow fibrosis patients. This suggests that some peripheral CD34⁺ cells may originate from the spleen rather than the bone marrow.⁴¹

Clonal chromosomal abnormalities were found in as many as 57% of patients with PMF. The most common ones reported include 13q⁻, 20q⁻, +8, -9, 12p⁻, and abnormalities of chromosomes 1 and 7. Although numerous JAK pathway mutations have been identified in patients with PMF, no single hallmark mutation has been identified for the disease. The *JAK2* (V617F) mutation was reported in 56% of the patients with PMF. Granulocytes of patients with PMF had the *JAK2* (V617F) mutation that correlated with the activation of cell signaling and might be related to the abnormal trafficking of CD34⁺ cells with an increased CD34⁺ count in the peripheral blood. The *JAK2* (V617F) mutation may constitutively activate granulocytes that mobilize CD34⁺ cells. This results in higher CD34⁺ cell count, which correlates with progression to marrow fibrosis. Several other mutations have been observed, including *CALR* mutation found in approximately 25% of patients with myelofibrosis.

While PV and ET are characterized by hyperproliferation, PMF is characterized by ineffective erythropoiesis and ineffective megakaryopoiesis, suggesting that additional genetic abnormalities may play a role in the pathogenesis of PMF. However, because fibroblasts are not part of the clonal

process and do not share the same clonal chromosomal abnormalities, it could be suggested that bone marrow fibrosis may be a secondary reaction.

ADVANCED CONTENT

Patients with PMF also have high levels of circulating inflammatory cytokines, such as interleukin-6, which likely contribute to a hypermetabolic state and cytokine-mediated systemic symptoms. A number of cytokines are involved in the pathogenesis of fibrosis in PMF. These are all secreted from platelets and megakaryocytes, although in some instances they may also be secreted from other cellular or stromal sources. These include transforming growth factor B (TGF- β), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF). A specific importance is given to TGF- β since it is capable of promoting secretions of type I and type III collagen. Elevated levels of PDGF are found in all patients with IMF and are released from platelets and megakaryocytes undergoing intramedullary death. PDGF stimulates fibroblast proliferation and collagen secretion. The bFGF moderates megakaryocyte-stromal cell interaction resulting in proliferation of stromal cells such as fibroblasts. In general, *JAK2* (V617F) as well as *MPLW515* mutations have been reported to affect cytokine signaling, survival, and G1-S cell-cycle transition through a constitutive activation of JAK-STAT signaling.⁴²

Thrombopoietin promotes megakaryocyte growth and development. However, neither mutations in the thrombopoietin receptor nor autocrine stimulation of thrombopoietin have been shown to play a role in the pathogenesis of PMF. The presence of activating mutations affecting the thrombopoietin receptor MPL (*MPLW515L* and *MPLW515K*) in 5% to 7% of PMF and 2% to 4% of ET patients is in agreement with this assumption.⁴³

Clinical Findings

PMF is a chronic progressive disorder and patients may be symptom-free for many years. New or increasing splenomegaly is considered to be a marker of disease progression in PMF. One-third of PMF patients older than the age of 60 are asymptomatic at presentation. Diagnosis is made after routine physical examination and the incidental finding of unexplained splenomegaly, abnormal peripheral blood results, or both. With progression of extramedullary hematopoiesis, splenomegaly and sometimes hepatomegaly is present. Splenomegaly related to PMF is present in 85% to 99% of patients at diagnosis and can be massive in about 10%. Splenomegaly related symptoms include left upper quadrant pain, early satiety, and even left shoulder pain. Palpable hepatomegaly is present in 40% to 70% of the cases (Fig. 19-7). Although hepatomegaly is found in about 50% of patients, it is not generally excessive but may be accompanied by mild to moderate jaundice, ascites, or both. Portal hypertension may develop because of increased splanchnic flow due to splenomegaly



FIGURE 19-7 Hepatosplenomegaly, a characteristic finding in patients with idiopathic myelofibrosis with myeloid metaplasia (IMF/MM).

and/or intrahepatic obstruction associated with extramedullary hematopoiesis in the liver (Fig. 19-8). Rapidly developing extramedullary hematopoiesis in the liver can cause abnormal liver function, jaundice, portal hypertension, and hepatic failure.

Metabolic consequences of myelofibrosis are fever, anorexia, weight loss, night sweats, pruritus, and bone pain. Extramedullary hematopoiesis can occur in almost any organ and may be manifested by lymphadenopathy, pleural or pericardial effusion, or ascites. If the central nervous system is involved, patients will have an increased intracranial pressure, altered sensorium, motor and sensory impairment, and even cord compression due to extramedullary hematopoiesis in the epidural space. Additionally, if extramedullary hematopoiesis occurs within the pulmonary arterial circulation, it can result in severe pulmonary hypertension.

Anemia with weakness, pallor, lethargy, and dyspnea is common. Approximately 10% of patients present with a serious bleeding problem related to thrombocytopenia or thrombocytosis, qualitative platelet defects, or coagulation abnormalities. Symptoms of bleeding may be as minor as petechiae or ecchymoses or as serious as gastrointestinal

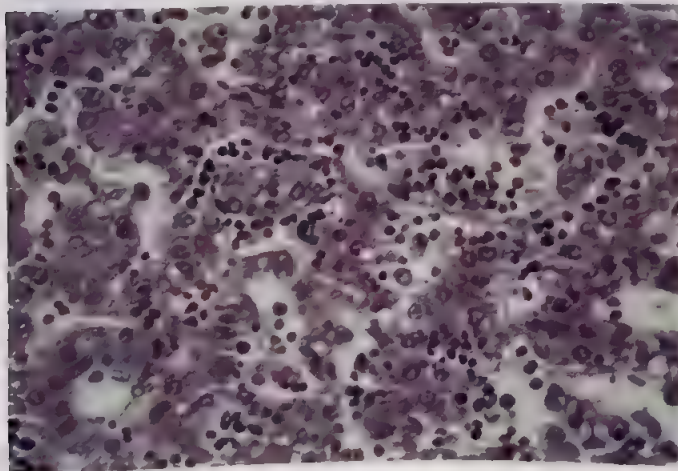


FIGURE 19-8 Extramedullary hematopoiesis in the liver of a patient with IMF.

bleeding. Infection related to the immune deficiency is common. Increased cellular turnover of nucleic acids can lead to hyperuricemia and secondary gout. Osteosclerosis, characterized by a diffuse or patchy increase in bone density and increased prominence of bony trabeculae on radiological studies, can be seen in PMF as well and may be associated with severe bone pain.

Investigators created the Myeloproliferative Neoplasms Symptom Assessment Form Total Symptom Score (MPN-SAF TSS), a 10-item instrument designed to assess the most representative and clinically relevant symptoms among patients with MPN. The form records the patient's assessment of the incidence and severity of these disease-related symptoms. This tool can be used to track symptoms over time and also to guide subsequent management decision.⁴²

Laboratory Testing and Results

The typical peripheral blood findings in PMF are related to both qualitative and quantitative cellular abnormalities. At diagnosis, normocytic normochromic anemia is present in 50% to 90% of patients. The cause of the anemia in PMF results from bone marrow failure, autoimmune hemolysis, ineffective or dyserythropoiesis, and from hypersplenism (dilutional anemia).

With disease progression, the morphological changes and the classic leukoerythroblastic blood picture become more apparent (Fig. 19-9). This includes the appearance of abundant nucleated red cells, immature granulocytes, and teardrop-shaped red cells in the peripheral blood (Fig. 19-10). Improvement or even normalization of red cell morphology can happen after splenectomy. Anemia is a result of ineffective erythropoiesis and hemolysis. Severe hemolytic anemia with marked reticulocytosis develops in 15% of cases, and it is generally direct antiglobulin test (DAT)-negative. However, in some cases, hemolysis is autoimmune in nature and may be DAT-positive with deposition of immunoglobulin IgG, IgM, or complement on the erythrocyte surfaces. Hypochromic, microcytic anemia may occasionally occur secondary to gastrointestinal bleeding. Additionally, megaloblastic anemia with macrocytes and occasionally hypersegmented neutrophils resulting from relative folate deficiency may also occur due to increased folate utilization.

The leukocyte count is variable in PMF. In about 50% of cases, the white blood cell (WBC) count exceeds $10 \times 10^9/L$; in approximately 35%, the WBC count is normal; and in nearly 15%, the WBC count is below normal. Marked leukocytosis (WBC greater than $30 \times 10^9/L$) is initially present in 11% to 13% of patients. On the other hand, leukopenia can develop in 8% of patients. With disease progression, an increase of immature myeloid cells is apparent. Rarely, leukocytosis may be extreme and mistaken for CML. The LAP score is typically normal. Serum levels of vitamin B_{12} are increased but not to the degree found in untreated CML. Eosinophilia and basophilia may be found frequently as in other myeloproliferative diseases.

The platelet count may be normal, elevated, or decreased in approximately 50% of IMF patients. Platelet counts range

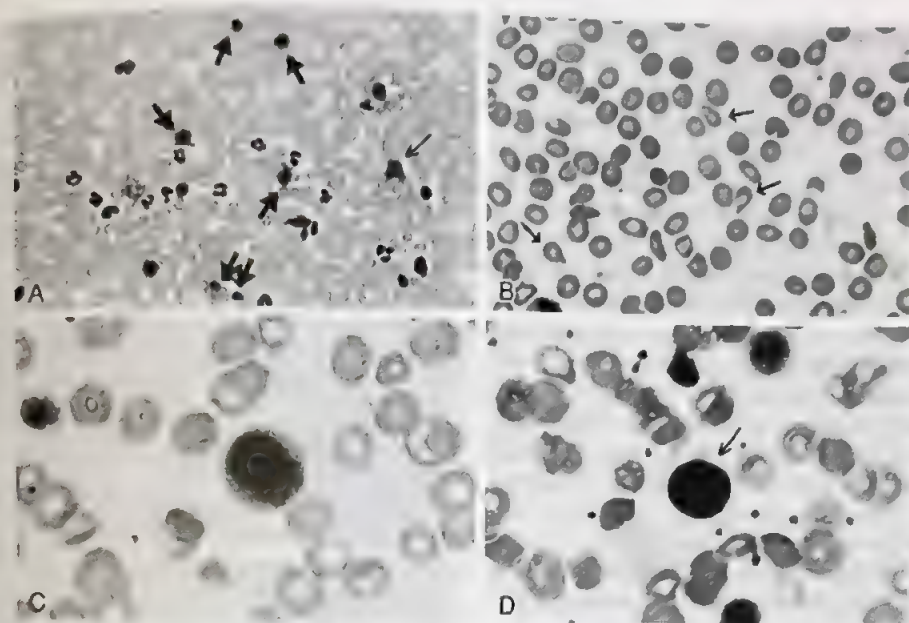


FIGURE 19-9 Leukoerythroblastosis, teardrop poikilocytosis, and abnormal platelet morphology associated with idiopathic myelofibrosis. *A.* Leukoerythroblastosis. Note the myeloblast at the large arrow and the numerous nucleated red blood cells at the small arrows. *B.* Teardrop poikilocytosis. *C.* Dwarf megakaryocyte (or micromegakaryocyte). This pathological alteration of a megakaryocyte may be found in any of the myeloproliferative disorders. Although often difficult to distinguish from cells of other lineages, observation of the marked cytoplasmic granularity and further comparison of this cytoplasm to that of other platelets present on the peripheral smear will aid in identification. *D.* Dwarf megakaryocyte. The cell at the pointer displays cytoplasmic blebs or budding, which is another characteristic of a micromegakaryocyte. Also note the giant platelets present on this peripheral blood smear.



FIGURE 19-10 Teardrop-shaped cells (arrows), peripheral blood in a patient with myelofibrosis.

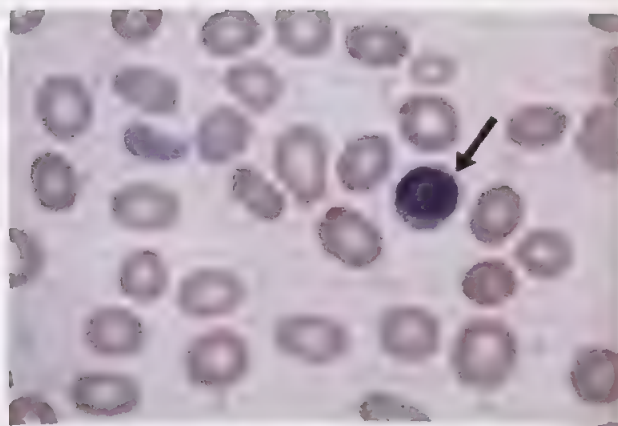


FIGURE 19-11 Micromegakaryocyte found on the peripheral blood smear of a patient with essential thrombocythemia.

from 450 to $1,000 \times 10^9/L$ at the time of diagnosis, but occasionally, counts in excess of 1 million are present. As the disease progresses, thrombocytopenia becomes increasingly prevalent. Giant dysplastic platelets are often seen, and megakaryocytic fragments or even dwarf megakaryocytes may be present in the peripheral blood (Fig. 19-11). The disturbed platelet morphology is reflected in abnormal platelet physiology with abnormal platelet functions present in as many as 50% of patients. Spontaneous platelet aggregation may also occur. The bleeding time is prolonged in up to 20% of patients but correlates poorly with the risk of bleeding. Although both thrombosis and bleeding may

occur in PMF, bleeding is more common, especially after splenectomy. As expected, after splenectomy, the number of immature WBCs, poikilocytes, and morphologically abnormal platelets increase.

Because of myelofibrosis, bone marrow aspirations are usually unsuccessful ("dry tap"). The reticulin and collagen fibrosis requires a needle biopsy for diagnosis. The bone marrow biopsy is hypercellular, with an increase in neutrophils and atypical megakaryocytes in the prefibrotic phase. Approximately 25% of patients present with the prefibrotic stage of disease. A left-shift in the differential counts may be observed without significant increase in myeloblasts

Erythroid precursors are easily found, but overall erythropoiesis is decreased. Megakaryocytes are morphologically abnormal with variations in size and “cloud-like” or “balloon-shaped” lobulations of the nuclei. Many naked megakaryocyte nuclei can also be seen. Reticulin fibrosis is minimal in the prefibrotic phase. In the fibrotic phase, the bone marrow cellularity is normal or decreased. Areas of hematopoiesis are separated by regions of loose connective tissue or fat. Reticulin fibrosis can be prominent with small islands of residual hematopoietic precursors. Atypical megakaryocytes often occur in clusters, sheets, or within dilated sinuses. Additionally, thickening of the bony trabeculae (osteosclerosis) may be seen. Grading the bone marrow fibrosis allows a better classification and categorization of patients related to their prognosis. Examples of cases with different degrees of fibrosis are shown in Figure 19–12.⁴³

Platelet dysfunction can cause hemostatic complications. Patients occasionally demonstrate prolonged PT and TT, as well as elevated levels of fibrin degradation products (FDP) and reduced levels of factors V and VIII. These findings, particularly with a low platelet count, indicate the presence of disseminated intravascular coagulation (DIC). Extramedullary hematopoiesis in the liver may result in hepatic dysfunction, which certainly contributes to the coagulation abnormalities (DIC, enhanced fibrinolysis). Hyperuricemia and elevated liver enzymes are found in one-third of the PMF patients.

Cytogenetic abnormalities are present in approximately 30% of cases at diagnosis, and this rate increases to approximately 90% after leukemic transformation. Chromosomal abnormalities are less frequent in younger patients, which may explain their better prognosis. Common findings include 13q-, 20q-, and trisomy 8 and abnormalities of chromosomes 1, 7, and 9. Complete or partial loss of chromosomes 5, 7, and 20 is associated with the treatment of PMF patients with chemotherapy. Although no specific cytogenetic abnormality is diagnostic for PMF, *JAK2* (V617F) is found in 20% to 40% of patients with PMF.

Differential Diagnosis

PMF must be distinguished from other diseases within the spectrum of the MPNs, as well as differentiated from fibrosis secondary to infiltrative disorders (see Table 19–1 and Box 19–2).

As discussed before, the prefibrotic stage of PMF clinically mimics ET. However, CML is considered most frequently in the differential diagnosis of PMF. In chronic cases of CML, there is marked leukocytosis, whereas in PMF, the WBC count is usually less than $30 \times 10^9/L$. Red cell morphology in CML is generally normal or may show a slight amount of anisocytosis and poikilocytosis compared with the significant teardrop poikilocytosis in PMF. The presence of the Ph chromosome and low LAP score are the strongest differentiating features that distinguish CML from PMF. Although differentiation of PMF from CML is not difficult, in rare atypical cases such as Ph-negative CML, it may be virtually impossible to differentiate from

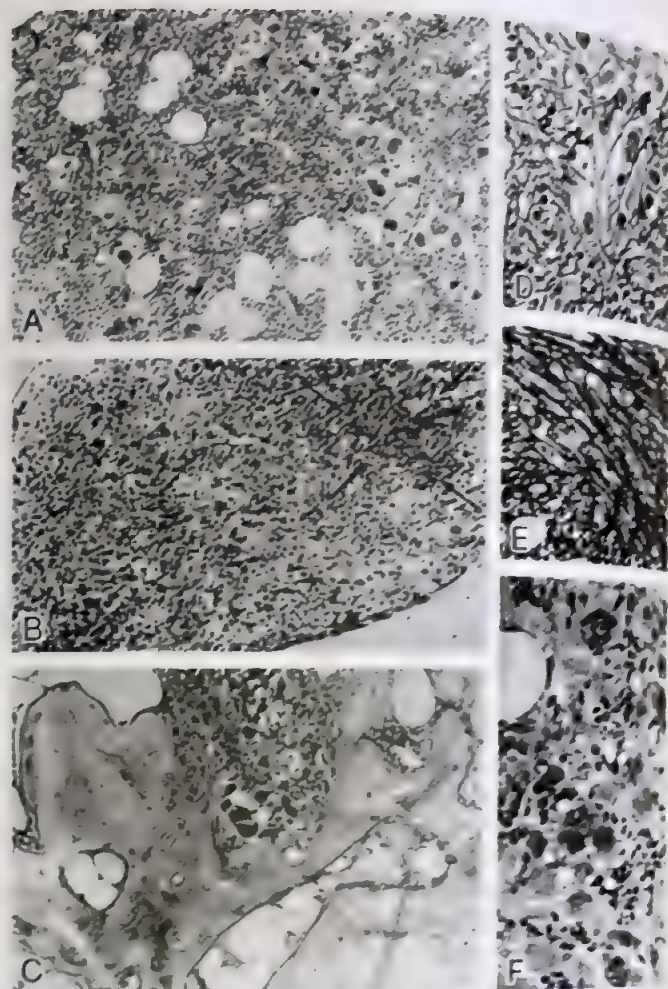


FIGURE 19-12 Histopathology of the bone marrow in idiopathic myelofibrosis. A. Early hyperplastic state without fibrosis. B. Advanced stage with a conspicuous increase in reticulin fibers, a still hypercellular marrow, and a lymphoid infiltrate (arrows). C. Late osteosclerotic state with endophytic bone formation, a residual cluster of hematopoiesis, and large areas of fatty tissue. D. Moderate degree of reticulin fibers surrounding atypical megakaryocytes in early IMF. E. Coarse bundles of obvious collagen fibers encompassing a few hematopoietic elements in terminal stages of IMF. F. Clusters of pleomorphic megakaryocytes displaying an abnormal maturation, and mitosis (arrowhead). A–C, magnification $\times 140$; D–F, $\times 350$. A–C and F, periodic acid–Schiff (PAS) stain; D and E, Gomori's silver impregnation. (From Thiele J, et al. Primary myelofibrosis-osteosclerosis, agnogenic myeloid metaplasia. *Am J Pathol*. 1989;94:30, with permission)

PMF with leukocytosis and minimal fibrosis (the cellular phase of PMF). Approximately 15% to 20% of patients with known PV undergo a transition to terminal myelofibrosis with marked anemia, bone marrow fibrosis, and progressive splenomegaly.

PMF must be differentiated from secondary causes of myelofibrosis. Box 19–2 lists marrow fibrosis secondary to infiltrative disorders and conditions secondary to nonmalignant disorders. The presence of tartrate resistant acid phosphatase (TRAP)-positive lymphocytes in the marrow and peripheral blood is typical of hairy cell leukemia and allows differentiation from PMF. Multiple myeloma with diffuse marrow plasmacytosis and the presence of serum and urine monoclonal protein is easily differentiated from PMF. Myelodysplastic (MDS) with marrow fibrosis can be difficult to differentiate from PMF. However, the marked splenomegaly, extramedullary hematopoiesis, and osteosclerosis are indications of PMF.

BOX 19-2 Differential Diagnosis of Myelofibrosis

- **Idiopathic Myelofibrosis (PMF)**
- **Other Chronic Myeloproliferative Disorders**
 - Chronic myelogenous leukemia
 - Polycythemia vera
 - Essential thrombocythemia
 - Transitional myeloproliferative disorders
- **Secondary to Infiltrative Disorders**
 - Metastatic carcinoma
 - Hematologic malignancies involving bone marrow
 - Acute leukemia
 - Myelodysplastic syndromes (preleukemia)
 - Hairy cell leukemia
 - Non-Hodgkin's lymphoma
 - Myeloma
- **Secondary to Nonmalignant Conditions**
 - Granulomatous disorders
 - Sarcoidosis
 - Tuberculosis
 - Histoplasmosis
 - Toxic exposure to chemicals
 - Hypo- and hyperparathyroidism
 - Osteoporosis
 - Vitamin D deficiency
 - Systemic lupus erythematosus
 - Systemic sclerosis

Treatment

The goal of therapy in PMF is to improve cytopenia and to reduce massive splenomegaly. Treatment of PMF usually involves the use of cytotoxic agents to control thrombocytosis and leukocytosis, and to improve splenomegaly. Multiple drugs have been utilized to control this progressive disease.

CRITICAL THINKING QUESTION

19-3 Why is splenomegaly so common in PMF patients?

ADVANCED CONTENT

Hydroxyurea, on average doses of 500 mg twice a day, can aid in reducing spleen size and control of thrombocytosis and leukocytosis. Treatment with hydroxyurea may be associated with worsening of anemia, which in some instances can be ameliorated by the concomitant use of erythropoietin. However, potential leukemogenicity of hydroxyurea is a big concern. Interferon- α has also been reported to have a hematologic response in hyperproliferative patients. Etanercept, a tumor necrosis factor- α blocker, was also promising in a pilot study. There was also a good response to the treatment with androgens (fluoxymesterone and oxymetholone).

Many patients with severe anemia require transfusion support. The use of erythropoietin has not generally been successful in patients with PMF, although some regressions have been reported in those with low erythropoietin levels. Rare patients with PMF have developed autoimmune hemolytic anemia that may respond to steroids and, occasionally, cyclosporine. Thalidomide has also been reported to have beneficial effects in some patients, improving anemia and thrombocytopenia, perhaps through its anti-TNF activity. Minimal to moderate activity has been seen in reducing splenomegaly.

Splenectomy is an option for patients with symptoms related to splenomegaly, such as left upper quadrant discomfort, transfusion-dependent anemia, refractory thrombocytopenia, hypercatabolic symptoms, or portal hypertension. However, the operative mortality can be significant, and postoperative thrombocytosis can occur leading to postoperative thrombosis and decreased survival. Blast transformation following splenectomy has been reported. Splenic irradiation can provide transient benefit and symptomatic relief for patients who are poor surgical candidates. However, it must be done carefully in low doses because it can occasionally be associated with severe and irreversible pancytopenia. Allogeneic stem cell transplantation remains the only curative treatment modality for PMF at present; unfortunately, it is often precluded by the patient's advanced age, other comorbidities, and lack of available donors.

ADVANCED CONTENT

At the present time, a sustained disease-modifying activity or durable remissions are not seen in PMF with the currently approved JAK inhibitors.⁴³ Therefore, it is crucial to improve the existing understanding of the disease and treatment-resistant mechanisms in PMF; research efforts are ongoing to develop novel JAK inhibitors or drugs with distinct mechanisms of action that offer a better side effect profile and tolerability in patients.⁴⁴ According to the National Comprehensive Cancer Network, existing guidelines for the treatment of patients with myelofibrosis are based on risk of death as well as symptom and signs. Patients who are determined to be higher risk should be referred to transplant. For those who are not candidates for transplant, JAK inhibitors or a clinical trial are recommended. Patients who are lower risk and asymptomatic should undergo observation or consider a clinical trial, while those who are lower risk and symptomatic should consider a clinical trial, ruxolitinib, ropeginterferon α -2a, or hydroxyurea.

To improve survival in PMF, transplant (allogeneic stem cells) is the best choice of treatment. Eligibility for transplant is judged by a comprehensive prognostic scoring system, MIPSS70 (Mutation-Enhanced International Prognostic Score System), which is based on mutations, cytogenetics, and clinical factors.

SUMMARY CHART

- Myeloproliferative neoplasms (MPNs) arise from a malignant transformation of a single multipotential stem cell that is committed to differentiation of granulocytes, erythrocytes, and platelets.
- MPNs are grouped together because of shared characteristics, the most important being panhyperplasia of the bone marrow, extramedullary hematopoiesis, bone marrow fibrosis, and predilection for leukemic transformation.
- Elevation of the hematocrit (above 58% in males and above 52% in females) is the most important hallmark of polycythemia vera (PV).
- Elevated red cell mass, splenomegaly, decreased erythropoietin, normal arterial oxygen saturation, and increased leukocyte alkaline phosphatase (LAP) are other important features of PV.
- 90% of patients with PV have a mutation (V617F) in the *JAK2* gene resulting in activation of downstream pathways (STAT5).
- Approximately 15% to 20% of patients with PV will enter the spent phase with marrow fibrosis, worsening splenomegaly, and pancytopenia.
- Treatment of PV involves therapeutic phlebotomy or use of cytotoxic myelosuppressive agents, or a combination of both.
- Increased secretion of erythropoietin has been implicated as the stimulus responsible for all cases of secondary erythrocytosis.
- The arterial oxygen saturation is often decreased in patients with secondary erythrocytosis.
- Relative erythrocytosis occurs when there is depletion in circulating plasma volume (causing increased hematocrit but normal red cell mass), and it is often seen in patients with dehydration.
- Familial polycythemia is the result of mutations in the erythropoietin receptor or mutations in the oxygen sensing pathway regulating erythropoietin production (Chuvash polycythemia).
- The platelet count is markedly elevated in essential thrombocythemia (ET), often to more than $1,000 \times 10^9/L$.
- Hemorrhage and thrombosis caused by dysfunctional platelets, splenomegaly, erythromelalgia, and neurological manifestations are clinical features of ET.
- ET must be differentiated from the many causes of reactive thrombocytosis.
- Symptomatic patients or high-risk patients should receive cytoreductive therapy with hydroxyurea.
- All patients with platelets $< 1,000,000/\mu L$ should receive aspirin if they have no antecedent history of bleeding.
- Important features of primary myelofibrosis (PMF) are anemia with teardrop poikilocytosis, leukoerythroblastic blood picture, marked bone marrow fibrosis, splenomegaly, and variable but often elevated platelet counts.
- Cytokines secreted by megakaryocytes and platelets stimulate bone marrow fibroblastic proliferation in PMF.
- Giant, bizarre platelets and micromegakaryocytes (dwarf megakaryocytes) may be seen in the peripheral blood in PMF.
- Progressive disease is manifested by extramedullary hematopoiesis with increasing splenomegaly, hepatomegaly, weight loss, cachexia, portal hypertension, and pancytopenia.
- Hydroxyurea may be helpful in controlling splenomegaly, leukocytosis, and thrombocytosis but does not alter the natural history of the disease.
- Splenic radiotherapy and splenectomy may be helpful in carefully selected patients.
- Allogeneic bone marrow (or allogeneic stem cells) transplant remains the only curative treatment.

CASE STUDY 19-1

A 74-year-old man presented with complaints of increasing weakness, night sweats, shortness of breath, easy bruising, and a fever of 10 days' duration. The patient had lost about 10 lb over a 6-month period and noted early satiety. On physical examination, he was pale, underweight, and had a fever of 103°F. Massive splenomegaly, moderate hepatomegaly, and pulmonary congestion were noted. Purpuric lesions were present on the upper extremities.

Initial laboratory studies disclosed the following values: WBC count: $30.5 \times 10^9/L$; RBC count: $2.9 \times 10^{12}/L$; Hgb: 8.3 g/dL; Hct: 25.8%; MCV: 89 fL; MCH: 28.6 pg; MCHC: 32.2%; platelet count: $650 \times 10^9/L$. The differential count revealed 45% segmented neutrophils, 6% band neutrophils, 20% lymphocytes, 9% monocytes, 3% eosinophils, 3% basophils, 4% metamyelocytes, 3% myelocytes, 2% promyelocytes, 5% "dwarf" megakaryocytes, and 15 nucleated red blood cells (NRBCs) per 100 WBCs. Erythrocyte morphology demonstrated anisocytosis and poikilocytosis, with prominent teardrop red cells, polychromasia, and basophilic stippling. Platelet number was increased, and platelet morphology was abnormal as evidenced by the presence of giant platelets and hyper- and hypogranulated platelets and megakaryocytic fragments. Other laboratory tests included reticulocyte count, 3.6%; LAP score, 132 (normal is 22 to 124); LDH, 3054 U/L; uric acid, 13.2 mg/dL; stool occult blood, negative; Ph chromosome, negative; and direct antiglobulin test (DAT), negative. A bone marrow aspirate was attempted several times but was unsuccessful because of a dry tap. The bone marrow biopsy revealed trilineage hyperplasia with many clumps of dysplastic atypical megakaryocytes. Extensive fibrosis was also noted. Bacterial and fungal bone marrow cell cultures were performed, and all results were negative.

COMMENT

The diagnosis of primary myelofibrosis with myeloid metaplasia was based on the classic findings of leukoerythroblastic anemia, marked splenomegaly, thrombocytosis with circulating megakaryocyte precursors, teardrop erythrocytes, and increased fibrosis of the bone marrow. Chronic myelogenous leukemia was excluded because the majority of these patients have the Philadelphia chromosome, a low LAP, a higher proportion of myelocytes and myeloblasts, and a bone marrow showing predominantly granulocytic hyperplasia. In addition, the red cell morphological changes (in particular teardrop red cells) are more prominent in myelofibrosis.

Of patients with known PV, 15% to 20% undergo a transition to PMF; however, because there is no prior history of PV, this disease can be ruled out. Granulomatous disorders

and acute leukemia can be excluded by careful scrutiny of the bone marrow and the negative microbiologic cultures. An increased platelet count and marked proliferation of bizarre megakaryocytes would be highly unusual in acute leukemia.

Another disorder considered in differential diagnosis is essential thrombocythemia (ET). Again, these patients rarely show the red cell abnormalities associated with fibrosis in the marrow and splenic hematopoiesis, and the bone marrow is easily aspirated. The platelet count in ET is almost always greatly elevated, often greater than $1,000 \times 10^9/L$, and immature granulocytes are rarely prominent.

Hydroxyurea was administered to this patient and continued over the course of 1 year. Blood transfusions were required every 3 to 4 weeks to counteract the impending anemia. Androgen therapy (danazol) was also initiated. The patient's condition gradually worsened, and it was evident that the chemotherapy was only mildly effective in decreasing splenic size. Splenectomy was performed in an attempt to ameliorate his anemia and relieve the constitutional symptoms of splenomegaly.

Four years after initial presentation, this patient developed acute myelogenous leukemia and underwent a rapidly progressive fatal course. This case illustrates a typical course of myelofibrosis. The median survival is approximately 5 years, and treatment often has little effect in prolonging the survival.

QUESTIONS

1. Referring to the WBC differential, is a "left shift" evident in this case? Why or why not?
2. Give reasons why the bone marrow aspirate resulted in a "dry tap" in this case.
3. What is the significance of the teardrop red cells?

ANSWERS

1. Yes. Evidence of a "left shift" in the WBC differential is indicated by the presence of 4% metamyelocytes, 3% myelocytes, and 2% promyelocytes.
2. A "dry tap" occurred during the bone marrow aspiration procedure done on this patient because of the extensive fibrosis that is a hallmark feature of myelofibrosis. The reticulin and collagen fibers that cause fibrosis form a tight network, locking in the marrow contents. As a result, the bone marrow sinusoidal blood is not aspiratable.
3. The teardrop cells are significant because they correlate with bone marrow fibrosis. The teardrop shape of the red cell occurs as the cell passes through the narrow, fibrotic sinusoids of the bone marrow and spleen.

CASE STUDY 19-2

A 58-year-old white man was admitted to the hospital with pain and swelling of the left arm suggestive of thrombophlebitis. He had presented to his physician 2 days earlier with complaints of pounding headaches, blurred vision, tinnitus, and generalized pruritus, especially after bathing. The patient had been treated for gout for the past 2 months. Family history is unremarkable for any hematologic disorders. The patient is a nonsmoker.

On physical examination, the patient's face appeared flushed, and the retinal veins were engorged. Several ecchymoses were apparent on the legs. The spleen tip was palpable three fingerbreadths below the costal margin (indicating moderate splenomegaly). No hepatomegaly or lymphadenopathy was observed.

A complete blood count revealed the following values: WBC count: $20.3 \times 10^9/L$; RBC count: $7.53 \times 10^{12}/L$; Hgb: 18.2 g/dL; Hct: 58.0%; MCV: 77 fL; MCH: 24.2 pg; MCHC: 31.4%; platelet count: $710 \times 10^9/L$. The differential count demonstrated 80% segmented neutrophils, 8% band neutrophils, 9% lymphocytes, and 3% monocytes. Red cell morphology was consistent with a microcytic, hypochromic classification.

Subsequent investigations were undertaken as part of the diagnostic work-up of the erythrocytosis. Determination of the red cell mass (utilizing the ^{51}Cr dilution method) was performed and found to be 41 mL/kg (normal male is 36 mL/kg or less). The plasma volume was 40 mL/kg. Arterial oxygen saturation was 94%. The serum iron was 30 mcg/dL (normal is 50 to 170) and total iron binding capacity (TIBC), 460 mcg/dL (normal is 250 to 450). Serum vitamin B_{12} was 925 pg/mL (normal is 205 to 876), and vitamin B_{12} -binding capacity was 2,600 pg/mL (normal is 1,000 to 1,022). The LAP score was 198, and the uric acid determination was 10.3 mg/dL. A bone marrow examination revealed 95% cellularity, with panhyperplasia and many large megakaryocytes. Iron stores were absent, and the reticulin content was slightly increased.

COMMENT

Several findings in the history and physical examination suggest a presumptive diagnosis of PV. The nonspecific symptoms of headache and blurred vision are a result of cerebral circulatory disturbances caused by hyperviscosity. Thrombotic episodes, such as the phlebitis recorded in this patient, are vascular manifestations resulting from the thrombocytosis in conjunction with the hyperviscosity and increased blood volume. The facial plethora and engorged retinal veins are findings associated with conjunctival and mucosal blood vessel congestion. Generalized pruritus occurs in 30% of patients with PV and is related to hyperhistaminemia. The lack of cardiac or respiratory abnormalities and the presence of normal arterial saturation are helpful

in ruling out secondary erythrocytosis. The splenomegaly noted is a frequent finding in myeloproliferative disorders.

The most important clinical findings supportive of PV are the elevation of the Hgb and Hct, increased red cell mass, and normal plasma volume. Further, evidence of trilineage involvement, leukocytosis, and thrombocytosis, in addition to erythrocytosis and bone marrow panhyperplasia, strongly suggests a diagnosis of PV. Abnormal elevation of the vitamin B_{12} and B_{12} -binding proteins, uric acid, and LAP are consistent with a myeloproliferative process and are helpful in establishing a diagnosis of PV. The low serum iron and absence of iron stores indicate concomitant iron deficiency. In most patients, this is attributed to occult gastrointestinal blood loss and defective platelet function.

This patient fulfills all the diagnostic criteria for PV set forth by the WHO. Because this 58-year-old patient has an elevated Hct and platelet count and is symptomatic (thrombophlebitis), both phlebotomy and myelosuppressive therapy were initiated. Colchicine and allopurinol were used to control the gout experienced by this patient. Pruritus was a persistent complaint despite the management of erythrocytosis by phlebotomy and hydroxyurea. Cyproheptadine was prescribed and found to be successful in controlling the pruritus.

QUESTIONS

1. What laboratory parameters listed in this case indicate a microcytic, hypochromic process?
2. If an erythropoietin level were ordered on this patient, would the expected result be normal, increased, or decreased?
3. What is the reason for the splenomegaly in this patient?

ANSWERS

1. The following red cell parameters indicate a microcytic, hypochromic process in this patient: MCV, 77 fL; MCH, 24.2 pg; MCHC 31.4%. Iron deficiency causes this process and results from the tremendous increase of iron required for the excessive erythropoiesis seen in PV and is often complicated by blood loss.
2. The expected erythropoietin level in this patient would be decreased, indicating autonomous production of red cells by the bone marrow. This is a particularly cost-effective diagnostic tool for differentiating PV from other secondary and relative causes of erythrocytosis. Greater than 95% of patients with PV exhibit the *JAK2* mutation (V617F), making this a very useful diagnostic test as well.
3. The characteristic mild to moderate splenomegaly seen in individuals with PV is attributed to extramedullary hematopoiesis. Splenic enlargement is seen in about 75% of patients and is an important finding.

CASE STUDY 19-3

This 35-year-old white woman initially presented with thrombocytosis (platelet count, $1,200 \times 10^9/L$) discovered on routine physical examination. Her WBC and Hgb values were normal. The history was unremarkable except for occasional epistaxis and minor bruising. She was advised to have a routine follow-up examination and CBC every 3 months and, despite a continually elevated platelet count, remained asymptomatic for 3 years. At that time, she was seen by her physician with complaints of dizziness, visual disturbances, and erythromelalgia. She had also had recent dental surgery and experienced a major perioperative bleeding episode. Mild splenomegaly was noted. Her platelet count was $2,500 \times 10^9/L$. Other laboratory values were as follows: WBC count: $18.5 \times 10^6/L$; Hct: 28.5%; prolonged bleeding time; reduced platelet adhesion; and defective platelet aggregation with epinephrine. Bone marrow biopsy demonstrated megakaryocytic hyperplasia with massive platelet clumping. Erythroid and myeloid hyperplasia, as well as a mild increase in reticulin content, were also observed.

Plateletpheresis was performed to rapidly reduce the marked thrombocytosis. The patient was treated with the myelosuppressive agent hydroxyurea in dosages varying from 1 g/day to 500 mg five times per week, depending on the platelet counts. The bleeding and vaso-occlusive symptoms were resolved, and coagulation abnormalities were corrected. Close follow-up is necessary for this patient to ensure a continued beneficial clinical and laboratory response.

COMMENT

This case highlights the common findings in essential thrombocythemia (ET); namely, marked increased platelet counts, thrombohemorrhagic events, splenomegaly, and bone marrow megakaryocytic hyperplasia. Although this is primarily a disease of upper-middle-age (50 to 70 years), a second population of younger, predominantly female patients exists. Two-thirds of patients are asymptomatic, as was this patient initially. With the advent of automated cell counters that routinely generate platelet counts, asymptomatic patients are being discovered more frequently.

The erythromelalgia noted in this patient represents one of the most characteristic vaso-occlusive manifestations. Prolonged bleeding after trauma or surgery is a common finding related to platelet dysfunction.

In an asymptomatic young patient with platelet counts below $1,000$ to $1,500 \times 10^9/L$, it is advisable to withhold myelosuppressive therapy, because these patients do well for many years untreated. When a patient requiring surgery presents with markedly increased platelet count and hemorrhagic complications, plateletpheresis will lower the

platelet count dramatically. In addition, myelosuppression is necessary to control the hyperproliferative process.

Causes of reactive thrombocytosis, such as iron deficiency anemia, malignancy, inflammatory disorders, splenectomy, and so on, are generally easy to exclude based on the clinical and hematologic features of the individual patient. To reliably exclude the other chronic MPNs, the WHO guidelines should be followed. To distinguish a patient with ET from an iron-deficient PV patient, serum iron, TIBC, ferritin, and bone marrow iron stains are usually sufficient. A trial of oral iron is rarely required. In patients with anemia, splenomegaly, thrombocytosis, and leukocytosis, the presence of the Ph chromosome is unequivocal evidence of CML.

The outlook for long-term survival in ET is encouraging as long as appropriate measures are taken to minimize thrombohemorrhagic complications. Many patients can tolerate markedly increased platelet counts for years without any complications. The introduction of plateletpheresis has allowed dramatic response in life-threatening or urgent surgical situations. In addition, hydroxyurea has proved to be an effective chemotherapeutic agent.

QUESTIONS

1. Why did this patient experience a major perioperative bleeding episode when her platelet count was $2,500 \times 10^9/L$?
2. Erythromelalgia can progress to what clinical manifestation?
3. What is the reason for the megakaryocytic hyperplasia seen in the bone marrow biopsy?

ANSWERS

1. Although the platelet number is elevated in ET, the platelets function abnormally. Severe thrombocythemia (greater than $1,500 \times 10^9/L$) can be associated with deficiency of the large von Willebrand multimers and resulting hemorrhage. Besides abnormal platelet function, hemorrhage in individuals with ET has been attributed to thrombosis with infarction, ulceration of the infarction, and subsequent bleeding.
2. The erythromelalgia of the toes, feet, and fingers seen in ET patients can progress to cyanosis and/or necrosis of the extremities. This toxic effect is caused by the metabolites of platelet arachidonic acid.
3. The megakaryocytic hyperplasia of ET results from a neoplastic clonal disorder of multipotential stem cells, which gives rise to excessive numbers of circulating platelets. This disease can be contrasted with the various disorders that can be associated with a reactive or nonneoplastic thrombocytosis.

REVIEW QUESTIONS

- What is the origin of myeloproliferative neoplasms?
 - Fibroid infiltration of major organs
 - Neoplastic transformation of multipotential stem cells
 - Widespread deterioration of cellular function
 - Splenic sequestration of normal blood cells
- Which myeloproliferative neoplasm is Ph(+)?
 - Polycythemia vera
 - Chronic myelogenous leukemia
 - Essential thrombocythemia
 - Primary myelofibrosis
- Which condition would present with increased Hct values, but normal RBC?
 - Polycythemia vera
 - Relative erythrocytosis
 - Secondary polycythemia
 - Essential thrombocythemia
- Increased EPO levels are seen in which of the following?
 - Polycythemia vera
 - Relative erythrocytosis
 - Secondary polycythemia
 - Absolute erythrocytosis
- Which of the following is the most common complication in polycythemia vera?
 - Myocardial infarction
 - Itching
 - Gout
 - Thrombosis
- What are the laboratory findings in polycythemia vera?
 - Decreased hematocrit; increased RBCs and granulocytes; decreased platelets
 - Increased hematocrit; increased RBCs, granulocytes, and platelets
 - Normal hematocrit; normal RBCs; increased granulocytes and platelets
 - Increased hematocrit; increased RBCs; decreased granulocytes and platelets
- What is the expected erythropoietin value in polycythemia vera?
 - Normal
 - Increased
 - Decreased
 - Variable
- The most striking peripheral blood finding in polycythemia vera is
 - Increased RBCs
 - Increased basophils
 - Increased monocytes
 - Decreased RBCs
- Which of the following findings align with a diagnosis of essential thrombocythemia?
 - Plt count less than or equal to $450 \times 10^9/L$
 - BM biopsy showing proliferation of myeloblasts
 - Presence of *JAK2*
 - Meeting WHO criteria for other myeloid neoplasms
- What is the safest and least expensive treatment for patients with polycythemia vera?
 - High altitude
 - Decrease of iron levels
 - Therapeutic phlebotomy
 - Decrease of erythropoietin levels
- Which is a complication of essential thrombocythemia?
 - Skin rash
 - Splenomegaly
 - Thrombosis
 - Neuropathy
- Which of the following is a laboratory finding consistent with the diagnosis of essential thrombocytosis?
 - Thrombocytopenia
 - Thrombocytosis
 - Leukocytopenia
 - Decreased RBCs
- The hallmark clinical finding in primary myelofibrosis is which of the following?
 - Splenomegaly
 - Bruising
 - Weakness
 - Pallor
- Which of the following abnormally shaped RBCs is found in primary myelofibrosis?
 - Helmet cells
 - Target cells
 - Schistocytes
 - Teardrop cells

See answers at the back of this book

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Myelodysplastic Syndromes

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CHAPTER OUTLINE

Epidemiology, Etiology, and Pathogenesis

MDS and Precursor States:
Clonal Proliferative Diseases
Genetic Anomalies
Biological Characteristics of
Disease Progression
Ineffective Hematopoiesis

Clinical Findings

Prognosis

Morphologic Characteristics of Blood and Bone Marrow

Definitions of Specific Morphological Characteristics
Lineage Dysplasias

Classification of MDS Subtypes (WHO 2016)

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MDS With Multilineage Dysplasia
MDS With Ring Sideroblasts
MDS With Isolated del(5q)
MDS With Excess Blasts
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Laboratory Testing and Results

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Treatment

Supportive Care and Hematopoiesis-Improving Therapies
Therapies Oriented Toward Improving Survival

Myelodysplastic/Myeloproliferative Overlap Syndromes

Summary Chart

Case Study 20-1

Case Study 20-2

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 20-1 Summarize the clonal nature and stem cell origin of myelodysplastic syndromes (MDS).
- 20-2 Detail the clinical findings seen in patients with MDS.
- 20-3 Explain the revised International Prognostic Scoring System (IPSS-R).
- 20-4 Recognize the morphological features of MDS on examination of blood and bone marrow smears.
- 20-5 Differentiate the subtypes of MDS based on the World Health Organization (WHO) classification.

- 20-6 List the ancillary laboratory studies helpful in diagnosing MDS.
- 20-7 Differentiate between de novo and therapy-related MDS.
- 20-8 Name benign conditions that can share cellular features with MDS.
- 20-9 Contrast supportive vs. curative therapies for MDS.
- 20-10 Describe a myelodysplastic/myeloproliferative disorder.

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal hematologic malignancies characterized by peripheral blood cytopenias, ineffective hematopoiesis, dysplastic blood cells, and a propensity to transform into acute leukemia. MDS were first described in the 1930s as preleukemic anemia. In 1982, the French–American–British (FAB) Morphology Cooperative Group classified these syndromes into five distinct entities according to specific morphological criteria. The World Health Organization (WHO) modified this classification, incorporating biological, cytogenetic, and molecular features that help better categorize these disorders.¹ The WHO classification is presented in detail later in this chapter.

The clinical outcome of patients with MDS is variable, ranging from relatively benign conditions that do not require therapy to rapidly progressive diseases associated with a very poor prognosis. The only curative treatment for MDS is allogeneic stem cell transplantation, a therapeutic modality available to only a minority of patients. The mainstay of treatment has historically been supportive care, but drugs aimed at correcting some of the defective pathways involved in the pathogenesis of MDS might offer new hope for certain patients.

This chapter summarizes the current knowledge of the pathogenesis, clinical features, and treatment of MDS, emphasizing the role of the laboratory investigation in the diagnosis.

of this important hematologic disorder. A listing of the common acronyms and abbreviations used throughout the chapter is presented in Box 20-1.

Epidemiology, Etiology, and Pathogenesis

MDS predominantly affects the elderly, with a median age at onset of 70 years old and an incidence of at least 20 per 100,000 in patients over 70.¹ MDS occurs at a lower frequency in younger adults and children. Men are slightly more frequently affected than women with the exception of MDS with isolated del 5q.

MDS and Precursor States: Clonal Proliferative Diseases

Significant progress has been made in understanding the molecular pathogenesis of de novo MDS, where certain mutations occurring in hematopoietic stem cells may attain some survival advantage and become a small and persistent clonal population, sometimes years before a hematologic malignancy arises. Often these mutations occur in regions of the genome that confer no change in the cell's maturation, function, or proliferation. These clones may quickly disappear. Occasionally a mutation may confer a survival advantage or induce cell proliferation. As those populations accumulate additional mutations over time that result in further increased proliferation, aberrant maturation or function, and resistance to cell death, preferential expansion of those clonal populations can lead to disruption of normal hematopoiesis² (Fig. 20-1).

With the identification of clonal mutations associated with myeloid neoplasms in up to 10% of elderly patients, the clearest risk factor for MDS is this age-related accumulation of

mutations.³ These age-related mutations occur as **somatic mutations**, meaning they are acquired in individual cells that may then undergo clonal expansion but are not present in the germline and cannot be inherited. Additional risk factors have been associated with the development of MDS, such as exposure to environmental and occupational products, particularly benzene and cigarette smoking.¹ Benzene, which is processed *in vivo* to hydroquinone, induces damage to hematopoietic progenitor cells that may lead to MDS. MDS can also occur as a late complication in patients treated with chemotherapy or radiation therapy that may induce mutations in hematopoietic progenitor cells; in this case, it is referred to as therapy-related MDS (t-MDS).

A number of familial syndromes including bone marrow failure syndromes are also associated with increased risk of MDS, both because of decreased ability to repair genetic damage, such as in Fanconi Anemia, but also because of the use of cytotoxic therapies.^{1,4} Whereas most cases of MDS arise with a background of age-related or therapy-induced somatic mutations, germline mutations in a number of the same genes, such as *RUNX1*, *DDX41*, or *ANKRD26*, may lead to a familial predisposition to MDS and AML.^{1,4} These cases of MDS are termed "myeloid neoplasms with germline predisposition" in the WHO Classification.

ADVANCED CONTENT

MDS and Precursor States: Clonal Proliferative Diseases

As high throughput sequencing approaches, such as massively parallel sequencing (also known as next generation sequencing or NGS), have become more common, shared somatic mutations have been identified between patients with no clinical findings, those with cytopenias without morphological or cytogenetic findings of MDS, and patients with MDS. Cases where patients have somatic mutations in genes that are otherwise seen in MDS or other myeloid neoplasms but have no peripheral blood cytopenias and no findings to support a myeloid neoplasm are termed **clonal hematopoiesis of indeterminate potential (CHIP)**. As with MDS, CHIP is significantly more common in elderly individuals due to age-related acquisition of somatic mutations.⁵ The risk of the hematopoietic clones in these patients expanding and/or acquiring additional mutations and thereby progressing to disrupted hematopoiesis and a true myeloid neoplasm depends on the size of the existing clone, but it is thought to be roughly 10 times higher than the risk of myeloid neoplasia in a patient without CHIP.^{2,6} Still, the majority of patients with CHIP do not develop a myeloid neoplasm. The stronger clinical significance in CHIP is an increased cardiovascular risk, which may be mediated by altered inflammatory responses.⁷

Patients with persistent cytopenias without another etiology and no other evidence of myeloid neoplasia, including no significant dysplasia or cytogenetic findings, are termed

BOX 20-1 Common Acronyms and Abbreviations Related to Myelodysplastic Syndromes

- alloSCT – allogeneic stem cell transplant
- AML – acute myeloid leukemia
- AML-MRC – acute myeloid leukemia with myelodysplasia related changes
- CCUS – clonal cytopenias of uncertain significance
- CHIP – clonal hematopoiesis of indeterminate potential
- FAB classification – French American British classification
- FISH – fluorescence in situ hybridization
- IPSS-R – revised international prognostic scoring system
- MDS – myelodysplastic syndrome
- MDS/MPN – myelodysplastic/myeloproliferative neoplasm
- MDS-EB – myelodysplastic syndrome with excess blasts
- MDS-MLD – myelodysplastic syndrome with multilineage dysplasia
- MDS-RS – myelodysplastic syndrome with ring sideroblasts
- MDS-SLD – myelodysplastic syndrome with single lineage dysplasia
- NGS – next generation sequencing
- t-MDS – treatment-related MDS
- WHO classification – World Health Organization classification

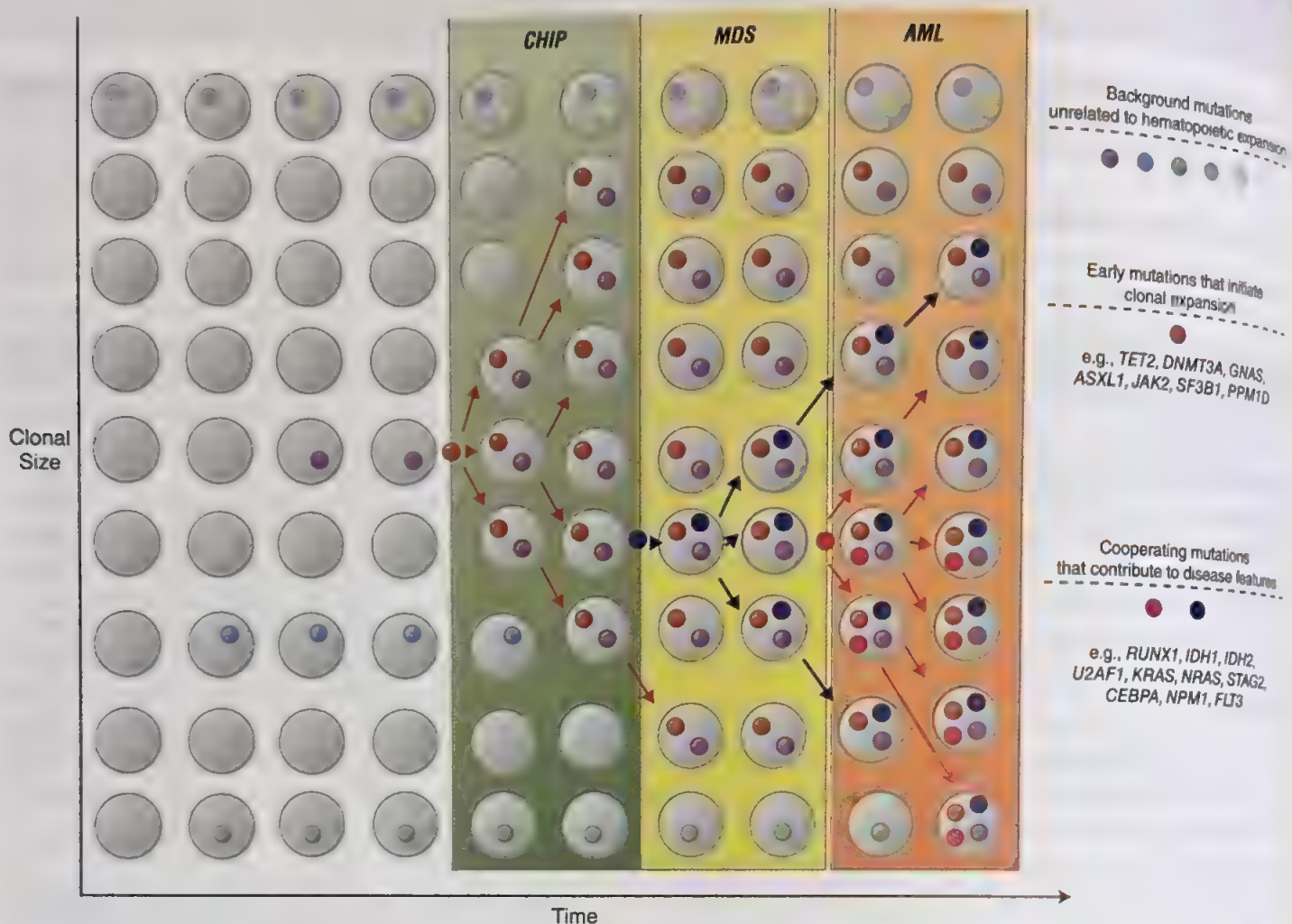


FIGURE 20-1 This schematic shows an example of the progression from clonal hematopoiesis of indeterminate potential (CHIP) to MDS and AML. CHIP may serve as a precursor state for hematological malignancies and in some cases to MDS or AML. Mutations that do not drive clonal expansion are shown either subsiding or persisting in a small percentage of hematopoietic cells (refer to colored circles indicating background mutations unrelated to hematopoietic expansion). Early mutations that drive clonal expansion (orange circles) appear in a larger hematopoietic clone and are shown subsequently acquiring additional mutations that lead to aberrant hematopoiesis (see mutations listed to the right under the orange circle). Cooperating mutations (listed to the right) that contribute to disease features are represented by the red and blue circles. Adapted with permission from Steensma, D. P., Bejar, R., Jaiswal, S., Lindsley, R. C., Sekeres, M. A., Hasserjian, R. P., & Ebert, B. L. (2015). Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes.

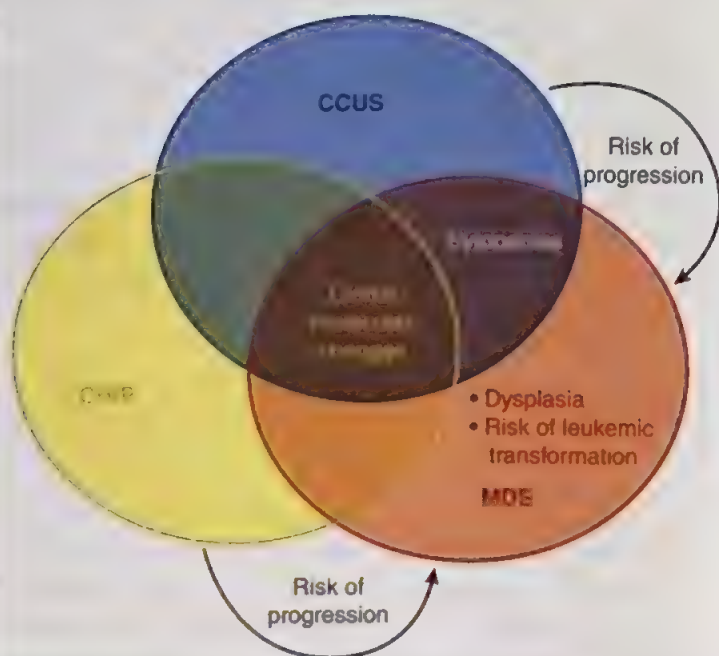


FIGURE 20-2 The relationship between CHIP, clonal cytopenias of uncertain significance (CCUS), and MDS are shown in this Venn diagram. All three entities are characterized by clonal molecular changes, but the presence or absence of cytopenias and dysplasia is needed to classify cases as CCUS or MDS. Both CHIP and CCUS show a risk of progression to MDS.

to have clonal cytopenias of undetermined significance (CCUS). The mutational profile of CCUS may show mutations in more genes and often larger clones than CHIP.⁴⁷ The findings in CCUS are also heterogeneous and can be further stratified into profiles with lower or higher risk of progression based on which genes show mutations.⁴⁸ A comparison of the findings in CHIP, CCUS, and MDS is presented in Figure 20-2. As our understanding of these precursor states improves, the molecular pathogenesis of MDS is also highlighted as a progression of accumulation of clonal aberrancies that drive dysfunctional hematopoiesis.

Genetic Anomalies

Clonality is a hallmark feature of myelodysplastic syndromes and can be demonstrated in several ways. A major development that has aided our understanding of the pathophysiology of MDS and precursor states is the ability to sequence many genes at once in massively parallel sequencing platforms. These panels allow us to identify different types of mutations in many different genes, often 50 or

more, at once. More than 80% of MDS cases show a mutation in at least one recurrently mutated gene and often show larger clone sizes and more mutations than CHIP or CCUS cases.^{8,9}

ADVANCED CONTENT

Frequently mutated genes include genes involved in histone modification (*ASXL1*, *EZH2*), epigenetic regulation and DNA methylation (*TET2*, *DNMT3A*, *IDH1*, *IDH2*), RNA splicing (*SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*), signaling networks and transcription factors (*TP53*, *RUNX1*, *NRAS*, *SETBP1*, *JAK2*, *ETV6*), and chromatid cohesion (*STAG2*).^{8,9} Frequently, multiple mutations are identified in genes with different functions, ultimately leading to the phenotype of abnormal maturation and ineffective hematopoiesis. The individual mutational profile has implications for classification, and in some cases, prognosis and therapy. One of the best examples of this is that *SF3B1* mutations are associated with the presence of increased ring sideroblasts, an important feature for WHO classification and better overall prognosis.⁹ Although the majority of individual gene mutations are not yet targetable by specific therapies, the overall profile can be helpful in risk stratification and therapy selection; for example, a less intensive treatment regimen may be appropriate in patients with an isolated *SF3B1* mutation.¹⁰

In addition to individual somatic gene mutations, clonality can also be assessed by **cytogenetic testing** where large deletions, duplications, and translocations can be identified by karyotype, fluorescence in situ hybridization (FISH), and chromosomal microarray techniques (see Chapter 35). Chromosomal abnormalities can be seen in 50% of de novo MDS cases and are even more frequent in t-MDS.⁹ The most frequent chromosomal abnormalities seen in MDS are¹

- Trisomy 8
- Monosomy 7 or del 7q
- del 5q
- del 20q
- Loss of chromosome Y
- Isochromosome 17q or t(17p)
- Monosomy 13 or del 13q

Chromosomal abnormalities are now incorporated into the WHO classification, where some chromosomal abnormalities are considered presumptive evidence of MDS in patients with cytopenias even without significant morphological dysplasia.¹ In addition to being necessary for appropriate classification, chromosomal abnormalities are also important for risk stratification and therapy selection, and are integrated into the revised International Prognostic Scoring System (IPSS-R) risk score to classify patients into prognostic groups.^{10,11}

Biological Characteristics of Disease Progression

ADVANCED CONTENT

The MDS phenotype changes as the disease progresses toward leukemia. Morphological examination of the peripheral blood and bone marrow may show increasing blasts. When blasts exceed 20% in the peripheral blood or bone marrow, the disease is considered transformed to acute myeloid leukemia with myelodysplasia-related changes (AML-MRC).¹ AML-MRC can be diagnosed if the patient has a history of MDS or myelodysplastic/myeloproliferative (MDS/MPN) overlap syndrome. In addition, AML-MRC can also be diagnosed if an AML shows characteristic MDS-related cytogenetic findings or multilineage dysplasia, and lacks a prior treatment history and recurrent cytogenetic abnormalities, which are hallmarks of specific AML subcategories, such as t(15;17) in acute promyelocytic leukemia.¹ As high-risk MDS with excess blasts and AML-MRC exist on a spectrum, the genetic aberrancies observed in AML-MRC are often also present in the preexisting MDS. In other cases, additional clonal cytogenetic and molecular aberrancies are seen as the percentage of blasts increases, suggesting that the disease clone has acquired additional aberrancies, termed clonal evolution, which have driven disease progression.^{12,13} As an example, in patients who do not have a *RUNX1* mutation at diagnosis, acquiring a *RUNX1* mutation is associated with leukemic transformation.¹⁴ MDS is thus one of the best examples of the multistep pathogenesis of neoplasia for hematologic cancers. The accumulation of different genetic alterations changes the phenotype of the cell from normal to clonal and dysplastic. Further changes confer a full-blown malignant phenotype, leading to the development of acute leukemia.

Ineffective Hematopoiesis

The *sine qua non* of MDS is peripheral blood **cytopenia or cytopenias**, meaning decreased red blood cells, white blood cells, or platelets in the blood. In conjunction with the typical normocellular or hypercellular bone marrow, this indicates **ineffective hematopoiesis** as the decreased peripheral blood counts are not due to decreased bone marrow precursors; however, hypocellular marrows can also be seen with MDS. The etiology of peripheral blood cytopenias includes aberrant apoptosis. The phenomenon by which each cell is able to trigger its own death is called **apoptosis** or programmed cell death, an essential part of tissue homeostasis. An abnormal decrease in the rate of natural apoptosis will lead to cell accumulation, whereas an increase in the rate of apoptosis will lead to decreased cellular output. In MDS, increased apoptosis and failed differentiation, where cells mature to a point and then undergo apoptosis, may contribute to ineffective hematopoiesis.^{15,16} Additional mechanisms for peripheral blood thrombocytopenia have been proposed and include autoimmune destruction, altered cell signaling, and functional defects in the peripheral blood cells and platelets.¹⁷

Clinical Findings

Most commonly, the presenting symptoms are those attributable to cytopenias. Clinical manifestations develop in relationship to the degree of anemia, neutropenia, or thrombocytopenia. Elderly patients with anemia may present with symptoms of cardiac failure, such as dyspnea on exertion. Fatigue, weakness, infections, and bleeding may also be presenting symptoms.³ Asymptomatic patients with MDS may also be identified following evaluation of cytopenias noted on CBC.^{3,18} Rarely, patients may present with systemic symptoms such as infection, arthralgias, fever, and cutaneous vasculitis.¹⁹ Abnormal findings on physical exam such as splenomegaly are less common in MDS and are more often associated with myeloproliferative neoplasms (MPN) or MDS/MPN overlap syndromes.

Prognosis

Because of the heterogeneous biology, behavior, and tendency to progress to AML among MDS, accurate assessment of prognosis is important in therapeutic decision-making. Several different prognostic models have been proposed, with the IPSS-R incorporating cytogenetics, bone marrow blast percentage, and degree of cytopenias²⁰ (Table 20-1). Overall, outcomes vary for survival, from more indolent cases with more than 10-year survival to rapid progression and median

survival of under 6 months.^{18,21} An important observation is that the majority of patients with low- and low-intermediate risk MDS still may die of causes related to their MDS, including infection and hemorrhage, rather than transformation to AML.^{18,22}

Morphological Characteristics of Blood and Bone Marrow

Definitions of Specific Morphological Characteristics

At the laboratory level, the diagnosis of a MDS is made by a combination of review of CBC data, careful morphological study of blood and bone marrow smears using the Wright-Giemsa stain, and the bone marrow biopsy specimen using hematoxylin and eosin (H & E) stain, with incorporation of ancillary studies to evaluate for clonality (Table 20-2). The iron content of both marrow aspirate and biopsy specimens is revealed by using Prussian blue (Perl's stain).

Dysplastic features, or abnormal morphological features, for each lineage are described in detail later in this chapter, termed **dyseryththropoiesis** in the erythroid lineage, **dysgranulopoiesis** in the granulocytes, and **dysmegakaryopoiesis** in megakaryocytes. Dysplastic features must be seen in at least 10% of the cells of the lineage to qualify as dysplasia. The review of these materials allows the distinction between

TABLE 20-1 Revised International Prognostic Scoring System (IPSS-R) for MDS Based on Percentage of Bone Marrow Blasts, Karyotype, and Blood Cytopenias

Score	0	0.5	1	1.5	2	3	4
Cytogenetics	Very good	-	Good	-	Intermediate	Poor	Very Poor
Bone marrow blast, %	≤2	-	>2%-5%	-	5-10%	>10%	-
Hemoglobin	≥10	-	8-10	<8	-	-	-
Platelets	≥100	50-100	<50	-	-	-	-
ANC	≥0.8	<0.8	-	-	-	-	-
Cytogenetics: "Very Good" = -Y, del(11q) "Good" = Normal, del(5q), del(12p), del(20q), double including del(5q) "Intermediate" = del(7q), +8, +19 i(17q), any other single or double independent clones "Poor" = -7, inv(3)/t(3q)/del(3q), double including -7/del(7q), complex: 3 abnormalities "Very Poor" = complex: >3 abnormalities							
Median Survival (years) by IPSS-R Score According to Age Group							
Overall Score/Risk	≤ 60 Years	>60-70 Years	>70-80 Years	>80 Years			
Very low = risk ≤ 1.5	Not reached	10.2	7.0	5.2			
Low = >1.5-3	8.8	6.1	4.7	3.2			
Intermediate = >3-4.5	5.2	3.3	2.7	1.8			
High = >4.5-6	2.1	1.6	1.5	1.5			
Very high = >6	0.9	0.8	0.7	0.7			

Adapted from: Greenberg PL, Tuechler H, Schanz J, Sanz G, Garcia-Manero G, Solé F, et al. Revised international prognostic scoring system for myelodysplastic syndromes. *Blood*. 2012 Sep 20;120(12):2454-65. PubMed PMID: 22740453; PubMed Central PMCID: PMC4425443.

TABLE 20-2 Morphological Findings in MDS

Features	Morphological Findings	
	Peripheral Blood	Bone Marrow Aspirate
Dyserythropoiesis	<ul style="list-style-type: none"> • Macrocytosis • Anisopoikilocytosis • Oval macrocytes • Dimorphic population 	<ul style="list-style-type: none"> • Megaloblastic changes • Multinucleation • Nuclear/cytoplasmic asynchrony • Karyorrhexis • Internuclear bridging • Nuclear fragments and budding • Ring sideroblasts • PAS positive erythroblasts
Dysgranulopoiesis	<ul style="list-style-type: none"> • Atypical neutrophil segmentation and hypercondensed chromatin • Pseudo-Pelger-Huet Anomaly • Occasional hypersegmentation • Hypogranularity and/or large granules 	<ul style="list-style-type: none"> • Myeloblasts, immature granulocytes • Occasional increase of monocytes and basophils • Nuclear/cytoplasm maturation • Asynchrony and maturation arrest • Hypogranularity and/or large granules • Auer rods
Dysmegakaryopoiesis	<ul style="list-style-type: none"> • Large or giant platelets • Hypogranular platelets • Micromegakaryocytes and megakaryocyte fragments 	<ul style="list-style-type: none"> • Normal or increased megakaryocytes, occasionally decreased • Micromegakaryocytes • Hypo- and monolobulation • Multiple separated nuclei "Botryoids or Pawn Ball"
Bone Marrow Biopsy	<ul style="list-style-type: none"> • Cellularity, evaluation of dysplasia of cell lineages, and abnormal localization of immature precursors • Cluster of megakaryocytes and degree of myelofibrosis 	

MDS subtypes based on established criteria. MDS are most often suspected in patients presenting with anemia (usually macrocytic or normochromic), with or without additional cytopenias.

An important caveat in the evaluation of dysplasia is that the presence of convincing morphological dysplasia in the blood or bone marrow does not confirm a diagnosis of MDS; benign conditions, including drug or medication effect, infections, nutritional deficiencies, some infections, and autoimmune disorders, can cause both dysplasia and cytopenias^{1,23} (Box 20-2). Obtaining a detailed clinical history and review of the patient's medications are critically important in avoiding the diagnostic pitfall of misdiagnosing a nonneoplastic condition as MDS. These pitfalls will be discussed in detail at the end of the chapter.

Blasts

Part of the MDS classification involves determining the number of blasts in the bone marrow at initial diagnosis. These blasts have the same characteristic features observed in other myeloid neoplasms: generally, they are large with high nucleus to cytoplasmic ratios and have fine glassy chromatin, with or without prominent nucleoli and azurophilic cytoplasmic granules (Fig. 20-3). They have a myeloid immunophenotype. The assessment of blasts for appropriate classification includes both the percentage of blasts as well as morphologic evaluation for the presence of Auer Rods, which are rod-shaped eosinophilic inclusions in blasts (Fig. 20-4). Although an increased blast percentage of 5% to 19% blasts in the peripheral blood or 10% to 19% blasts in the bone marrow are generally required for the diagnosis of MDS with excess blasts-2 (MDS-EB-2), the presence of any blasts with Auer Rods,

BOX 20-2 Benign Etiologies of Dysplasia

Chronic Infections

- Human immunodeficiency virus (HIV)
- Other viral infections

Nutritional Deficiencies

- Vitamin B₁₂ deficiency
- Folate deficiency
- Copper deficiency (or excessive Zinc)

Drugs or Toxins

- Alcohol abuse
- Arsenic
- Antitubercular drugs
- Chemotherapy drugs
- Immunosuppressant drugs

Systemic Disorders

- Autoimmune diseases

regardless of blast percentage, will qualify for a classification of MDS-EB-2.¹

Lineage Dysplasias

Dyserythropoiesis

Dyserythropoiesis refers to the ineffective development of normal red cells. In the peripheral blood, anemia is the most common presenting cytopenia, most often macrocytic or normocytic, with a decreased reticulocyte number.²⁴ Rarely, a

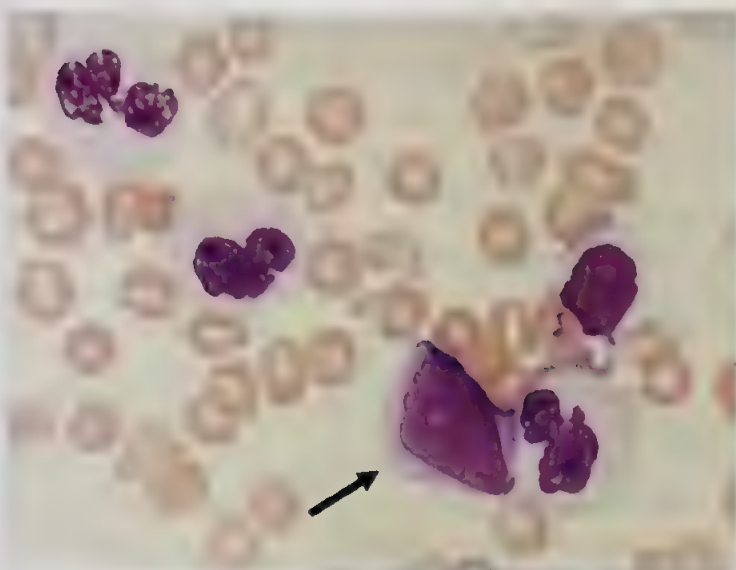


FIGURE 20-3 Blast morphologies (peripheral blood): a circulating blast shows fine chromatin with a prominent nucleolus and large nucleus with moderately abundant gray-blue cytoplasm (arrow); adjacent neutrophils also show dysplastic features with cytoplasmic hypogranularity.

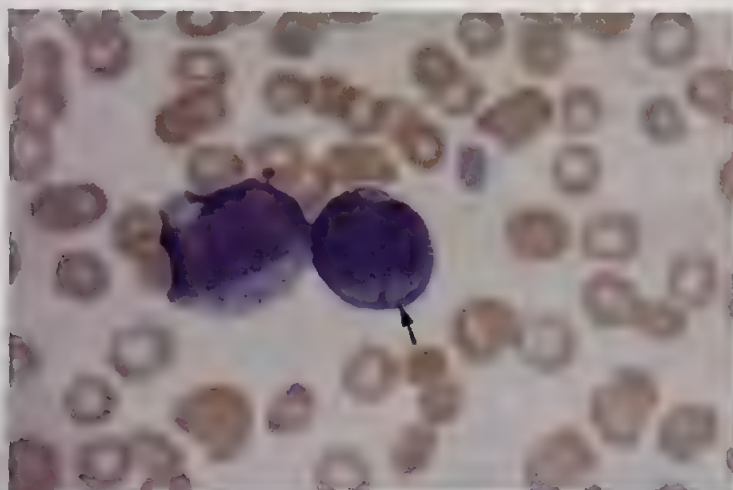


FIGURE 20-4 Blast morphologies (bone marrow aspirate): blast with an Auer rod (arrow) indicates that this case would be classified as myelodysplastic syndrome with excess blasts-type 2 (MDS-EB-2).

microcytic anemia can be seen in MDS associated with mutations in the *ATRX* gene³. Erythrocyte morphological characteristics that may be encountered are macrocytosis and a broad array of **anisopoikilocytosis** (variability in size and shape of red blood cells), with such features as basophilic stippling, a dual red blood cell (dimorphic) population (normochromic, hypochromic), Pappenheimer bodies, dacryocytes (teardrop cells), fragmented cells, elliptocytes, Howell–Jolly bodies, and acanthocytes (Figs. 20–5 to 20–8). These features are not specific for MDS but may reflect dyserythropoiesis.

In bone marrow erythroid precursors may show megaloblastoid changes with dense or fine chromatin with asynchronous cytoplasmic maturation and a wide variety of nuclear lobation abnormalities. Megaloblastoid changes alone are insufficient for qualifying as dysplasia.¹ Although normal erythroid precursors show perfectly round nuclei, dysplastic erythroids may show internuclear bridging, broad-based nuclear budding, karyorrhexis, and multinucleation¹

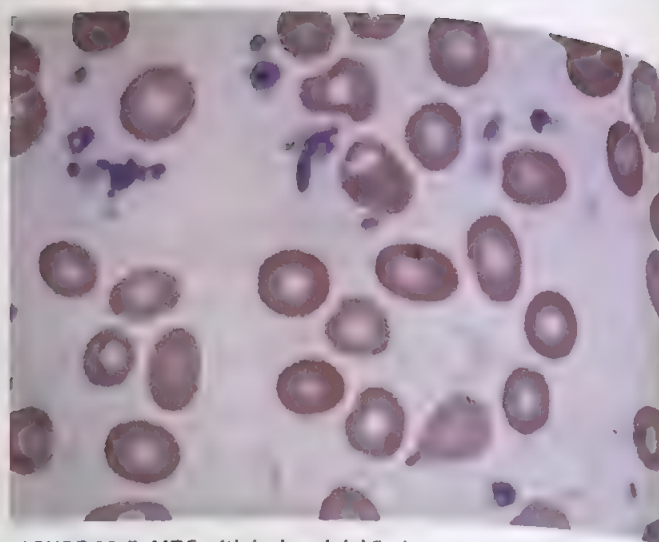


FIGURE 20-5 MDS with isolated del 5q (peripheral blood): red cell anisopoikilocytosis with oval macrocytes and thrombocytosis.

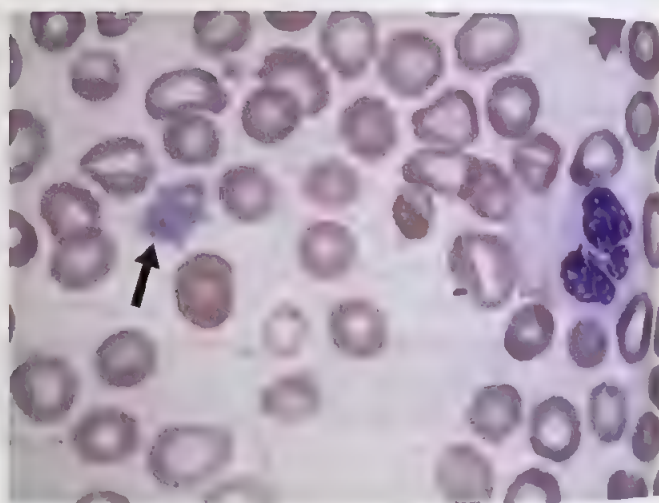


FIGURE 20-6 Dysplastic findings (peripheral blood): a hypogranular neutrophil with normal nuclear lobation is seen with background red cell anisopoikilocytosis and a giant platelet (arrow).

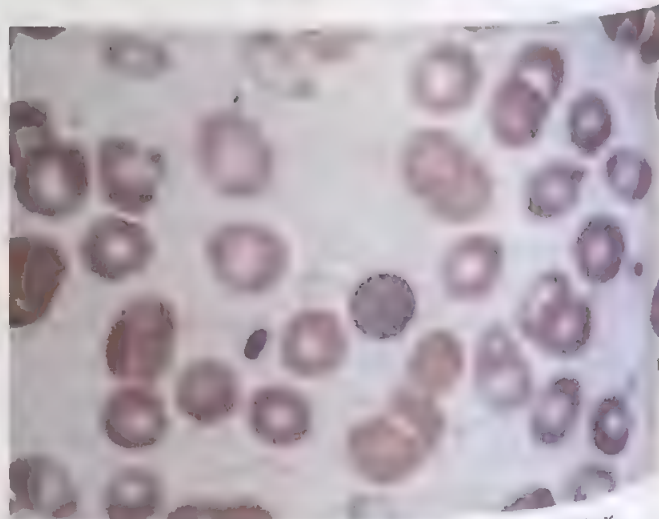


FIGURE 20-7 Myelodysplastic syndrome with ringed sideroblasts (MDS-RS) showing a dimorphic population of red blood cells including normocytic and macrocytic forms compared with the central lymphocyte (peripheral blood).

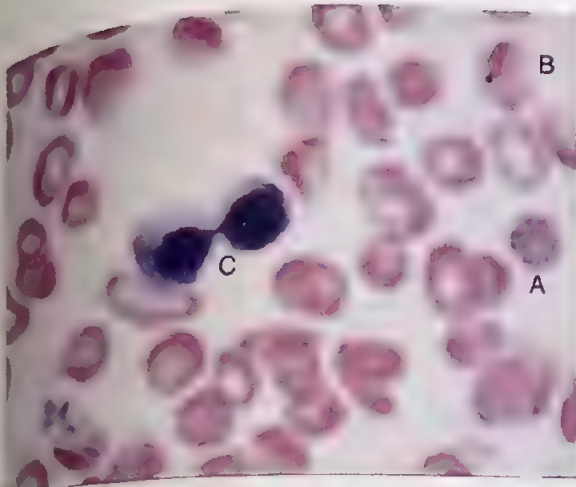


FIGURE 20-8 Spectrum of dyserythropoietic morphologies (peripheral blood): basophilic stippling (A), Howell-Jolly body (B), and erythroid precursors with internuclear bridging (C).

(Figs. 20-9 to 20-10). Cytoplasmic abnormalities may include vacuolization and aberrant PAS positivity.¹

Typically, about one-third of nucleated erythroid precursors contain some sideroblastic iron, which are present as small granules that are randomly distributed in the cytoplasm.^{25,26} A **ring sideroblast** is defined as having ≥ 5 granules in a perinuclear distribution that cover at least one-third of the nuclear rim or may form a complete ring around the nucleus²⁶ (Figs. 20-11 to 20-12). Even in the absence of other features of erythroid dysplasia by morphology, $\geq 15\%$ ring sideroblasts qualifies as dyserythropoiesis. Correlation with molecular findings is necessary, as $\geq 5\%$ ring sideroblasts qualifies as dyserythropoiesis in the setting of an *SF3B1* gene mutation.¹ Because the presence or absence of ring sideroblasts is required for appropriate classification in the WHO system, iron stains should be performed on the aspirate smears of all patients being evaluated for suspected MDS. Again, it is important to note that ring sideroblasts may be increased in nonneoplastic conditions including alcohol use, other toxins (including lead poisoning), drugs (including isoniazid), copper deficiency, and congenital sideroblastic anemia.¹



FIGURE 20-9 Spectrum of dyserythropoietic morphologies (bone marrow aspirate): multinucleated erythroid precursors with nuclear to cytoplasmic maturational dysynchrony (arrows).

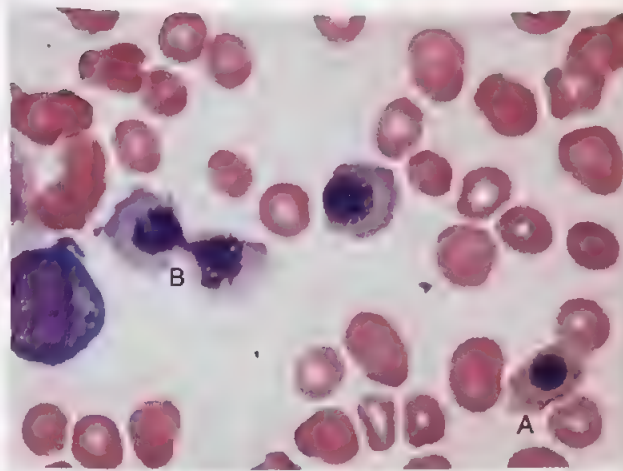


FIGURE 20-10 Spectrum of dyserythropoietic morphologies (bone marrow aspirate): basophilic stippling (A), erythroid precursors connected by internuclear bridging (B).

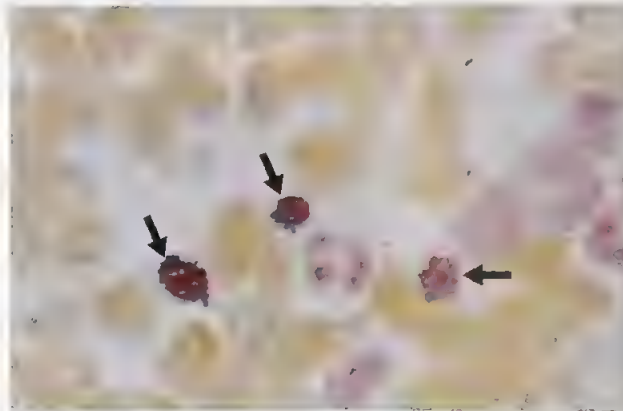


FIGURE 20-11 Ring sideroblasts (bone marrow aspirate, Prussian blue stain): three ring sideroblasts are seen, each with at least five iron granules localized around the nuclear rim covering at least a third of nuclear periphery (arrows).

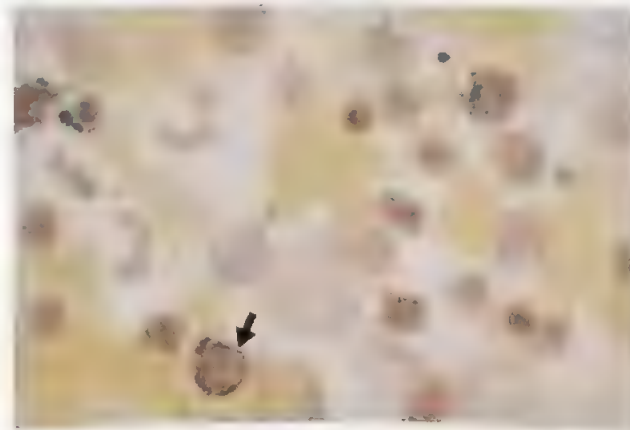


FIGURE 20-12 Ring sideroblasts (bone marrow aspirate, Prussian blue stain): many ring sideroblasts are seen.

Dysgranulopoiesis

Dysgranulopoiesis refers to the defective granulocytic development. In peripheral blood, neutropenia is found in almost 60% of patients.²⁷ The classic feature of dysgranulopoiesis in peripheral blood is a granulocyte with a bilobed nucleus and hypogranular cytoplasm (**pseudo-Pelger-Huet anomaly**), but other nuclear lobation anomalies including hypo- and hypersegmentation, pseudo-Chediak-Higashi granules, and small or giant forms can also be seen¹ (Figs. 20-13 to 20-15). Typically, both nuclear and cytoplasmic features of dysplasia are identified in cases with dysgranulopoiesis.

In the bone marrow, the nuclei of neutrophils often show variable degrees of hyposegmentation and hypogranularity (**pseudo-Pelger-Huet anomaly**) (Figs. 20-16 to 20-18). Other nuclear abnormalities include hypersegmentation and abnormal chromatin clumping.^{1,24}

Dysmegakaryopoiesis

Dysmegakaryopoiesis refers to the defective production of megakaryocytes and platelets. In the peripheral blood, moderate thrombocytopenia is present in approximately 40% of patients, occasionally being severe (less than $20 \times 10^9/L$)¹¹.

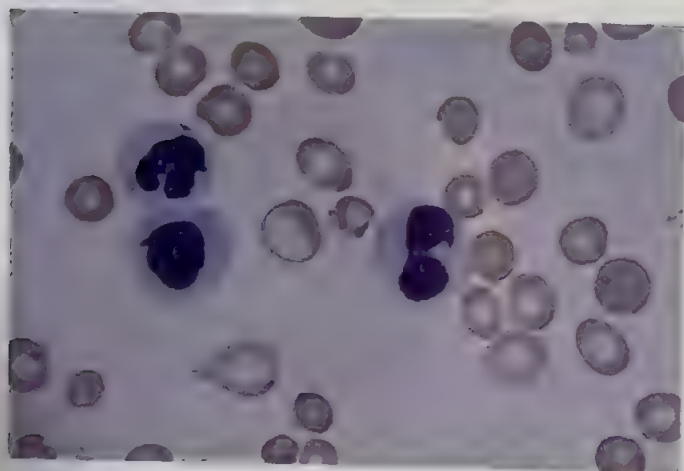


FIGURE 20-13 Dysgranulopoiesis (peripheral blood): three neutrophils all showing nuclear hyposegmentation and cytoplasmic hypogranularity (pseudo-Pelger-Huet anomaly when bilobed with hypogranularity).

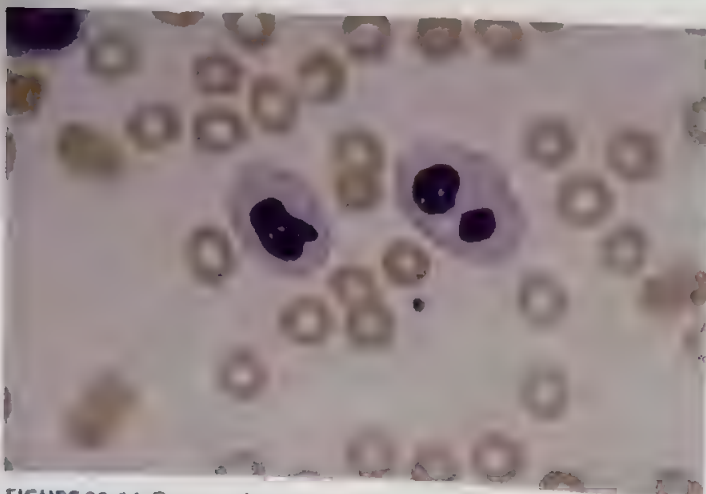


FIGURE 20-14 Dysgranulopoiesis (peripheral blood): two neutrophils with nuclear hyposegmentation and cytoplasmic hypogranularity.



FIGURE 20-15 Pelger-Huet Anomaly (peripheral blood): in true Pelger-Huet anomaly, a benign congenital condition, all neutrophils show nuclear hyposegmentation with normal cytoplasmic granulation; whereas in MDS, typically only a subset of neutrophils are dysplastic and show some combination of nuclear hyposegmentation and cytoplasmic hypogranularity.

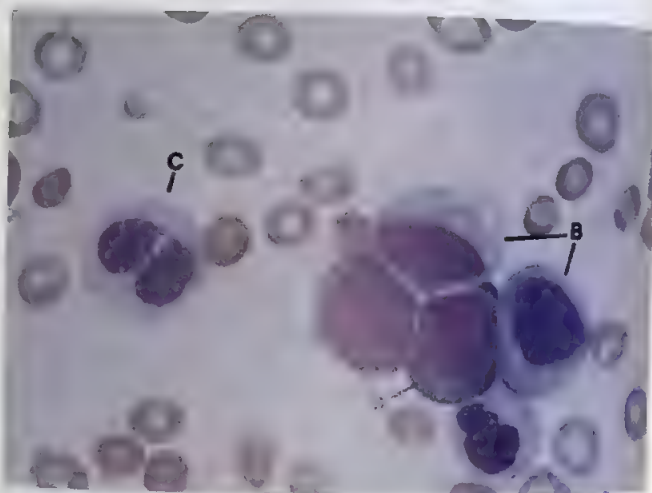


FIGURE 20-16 Dysplastic findings (bone marrow aspirate): blast cells (A), granulocytic precursors with hypogranular cytoplasm (B), and a hypoblast, hypogranular neutrophil (pseudo-Pelger-Huet cell) (C).

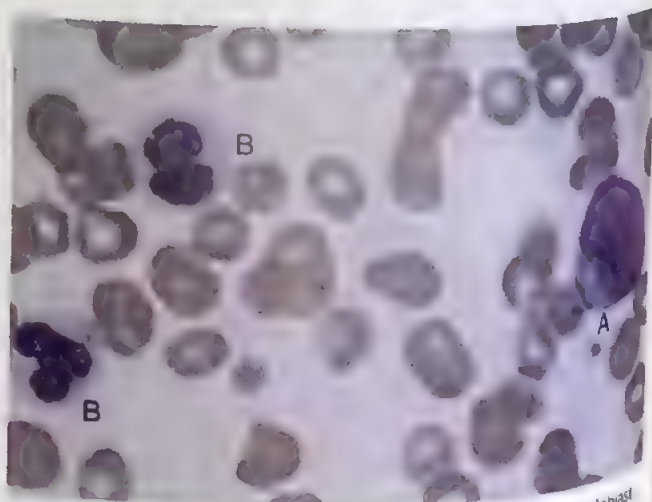


FIGURE 20-17 Dysgranulopoiesis (bone marrow aspirate): a myeloblast (A) is seen next to two hypogranular neutrophils (B).

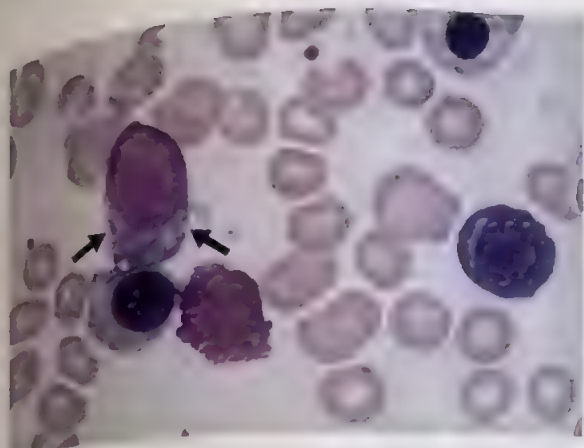


FIGURE 20-18 MDS-EB-2 a myeloblast is seen with two Auer rods (arrows) with a smudge cell below right and dyserythropoiesis below left. (bone marrow aspirate).

Thrombocytosis is a rare occurrence but may be seen in a third of cases of MDS with isolated $\text{del } 5q^1$ (Fig 20-5). One may find variable morphological abnormalities such as platelet gigantism, ballooning, and variable hypogranulation. Rarely, circulating micromegakaryocytes or bare megakaryocyte nuclei can be found in peripheral blood (Figs. 20-19 to 20-20).

In the bone marrow there are two characteristic morphological abnormalities of megakaryocytes in the bone marrow: the presence of **micromegakaryocytes** (dwarf or mononuclear megakaryocytes) with nuclear hypolobation, and megakaryocytes with multiple small nuclei detached from one another or separated by a thin strand of nuclear material and cytoplasmic hypogranularity ("pawn-ball" shape)^{1,24} (Figs. 20-21 to 20-24). These features are best appreciated in aspirate smears, whereas bone marrow biopsies may show similar features and nuclear hyperchromasia. In some cases, micromegakaryocytes may be hard to appreciate on core biopsies, but can

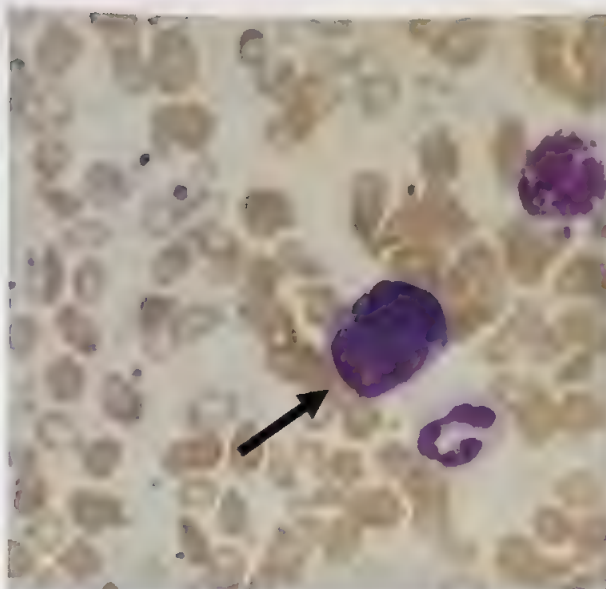


FIGURE 20-20 Rarely micromegakaryocytes or bare megakaryocytic nuclei can be observed in the peripheral blood.

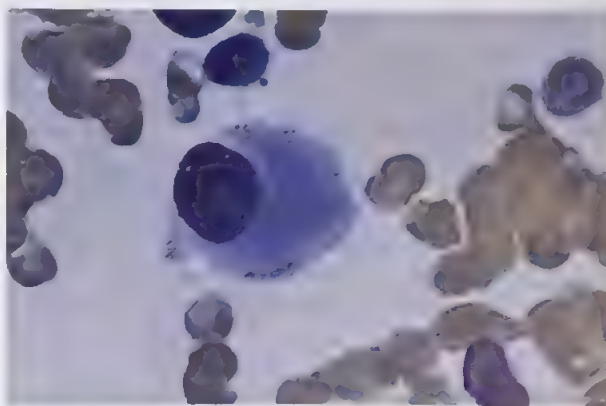


FIGURE 20-21 Dysmegakaryopoiesis: a dwarf megakaryocyte that is small with a monolobular nucleus. (bone marrow aspirate).

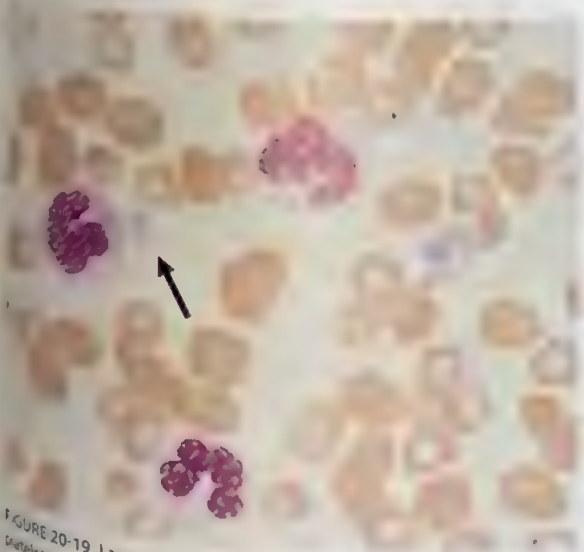


FIGURE 20-19 Large, hypogranular platelet (arrow) compared with the platelets with more normal granular at the right of the image (peripheral blood).

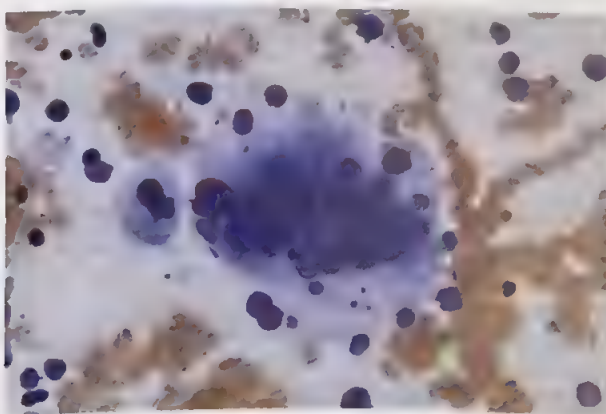


FIGURE 20-22 Dysmegakaryopoiesis: a large megakaryocyte with excessive and detached nuclear lobes ("pawn-ball" shape). Bone marrow aspirate.

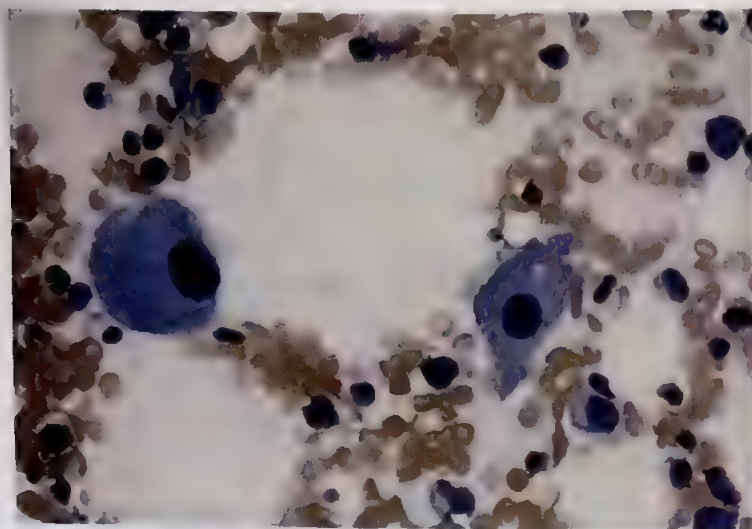


FIGURE 20-23 Dysmegakaryopoiesis: multiple small, hypolobate/monolobate megakaryocytes. (bone marrow aspirate).

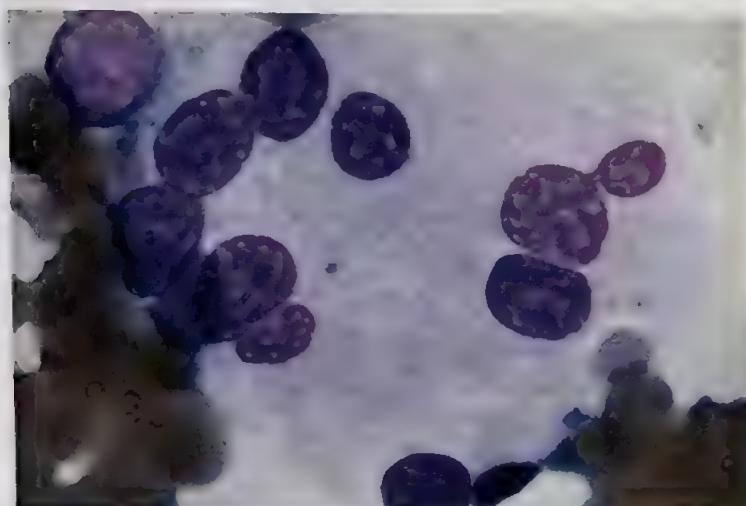


FIGURE 20-24 Dysmegakaryopoiesis: large megakaryocyte with hypogranular cytoplasm and detached nuclear lobes (bone marrow aspirate).

be identified with immunohistochemical stains for CD42b or CD61²⁴ (Fig 20-25 to 20-26).

CRITICAL THINKING QUESTION

20-1 Why could the presence of ringed sideroblasts be missed in routine bone marrow smear evaluation?

See answers to all Critical Thinking Questions at the back of this book.

Classification of MDS Subtypes

MDS classification has evolved considerably since the first introduction of the term preleukemia or refractory anemia several decades ago. Accurate classification is instrumental in helping investigators and clinicians to categorize patients into more homogeneous subsets, help predict outcome, and select therapeutic options for patients.

Under the 2016 WHO classification, MDS are classified into six distinct subtypes, which rely on CBC findings, morphological and cytogenetic information for appropriate classification.

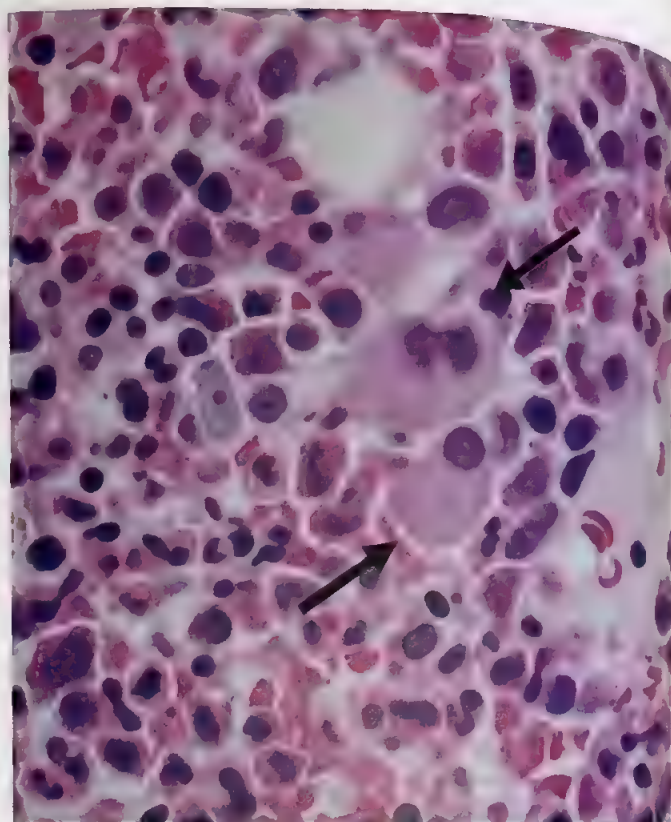


FIGURE 20-25 Dysmegakaryopoiesis (bone marrow core): increased dysplastic small megakaryocytes with nuclear hypolobation/monolobation are present (arrows).

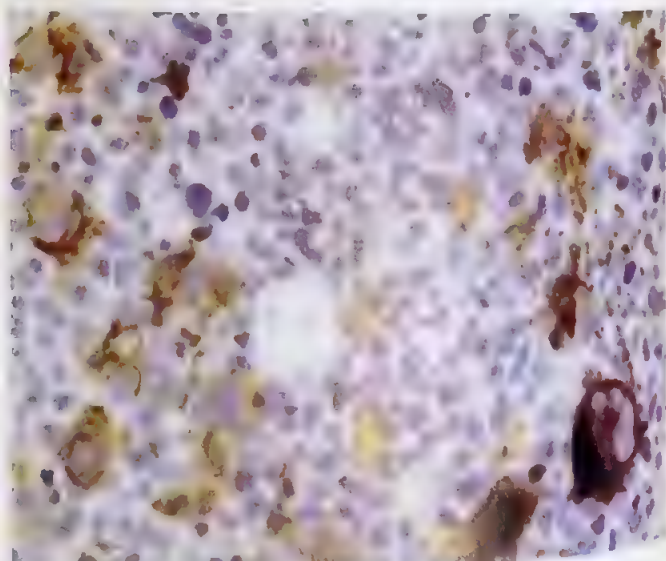


FIGURE 20-26 Dysmegakaryopoiesis (bone marrow core, CD61 stain): immunohistochemical stains for megakaryocytic markers, such as CD61 in this case, can highlight small, dysplastic megakaryocytes that would be difficult to appreciate on the hematoxylin and eosin stain.

It is important to note that, without cytogenetics, and in some cases without molecular testing, appropriate classification using the WHO guidelines is not possible, emphasizing the importance of ancillary testing. A summary of the diagnostic criteria for each subtype is presented in Table 20-3.

MDS With Single Lineage Dysplasia

As the name implies, MDS with single lineage dysplasia (MDS-SLD) involves a dysplasia of only a single lineage with cytopenias in 1 to 2 lineages. Typically, these patients present with anemia with or without an associated neutropenia.

TABLE 20-3 WHO Classification (2016) of MDS

Disease		Blood Findings	Bone Marrow Findings
MDS-SLD		Unicytopenia, typically anemia No or rare blasts (<1%) No Auer rods	Dysplasia in $\geq 10\%$ of cells in one lineage only <5% blasts No Auer Rods <15% ring sideroblasts*
MDS-MLD		Uni-, bi-, or pancytopenia No or rare blasts (<1%) No Auer Rods	Dysplasia in $\geq 10\%$ of cells in two or more myeloid cell lines No Auer Rods <15% ring sideroblasts* <5% blasts
MDS-RS	MDS-RS-SLD	Uni- or bicytopenia No or rare blasts (<1%) No Auer rods	Dysplasia in $\geq 10\%$ of cells in one lineage only <5% blasts in marrow No Auer rods $\geq 15\%$ ring sideroblasts*
	MDS-RS-MLD	Bicytopenia or pancytopenia No or rare blasts (<1%) No Auer rods	Dysplasia in $\geq 10\%$ of cells in two or more myeloid cell lines $\geq 15\%$ ringed sideroblasts* < 5% blasts No Auer rods
MDS with isolated del (5q)		Uni- or bicytopenias No or rare blasts (<1%) No Auer rods	Dysplasia in $\geq 10\%$ of cells in one or more myeloid cell lines <5% blasts No Auer rods Must demonstrate a del(5q) with or without one additional cytogenetic abnormality that cannot be del(7q) or monosomy 7
MDS-EB	MDS-EB-1	Uni-, bi-, or pancytopenia 2% to 4% blasts No Auer rods	Dysplasia in $\geq 10\%$ of cells in one or more myeloid cell lines 5% to 9% blasts No Auer rods
	MDS-EB-2	Uni-, bi-, or pancytopenia 5% to 19% blasts or any blasts with Auer rods	Dysplasia in $\geq 10\%$ of cells in one or more myeloid cell lines 10% to 19% blasts or any blasts with Auer rods
MDS-U	1% peripheral blood blasts	Uni-, bi-, or pancytopenia 1% peripheral blood blasts demonstrated on two consecutive occasions	Dysplasia in $\geq 10\%$ of cells in one or more myeloid cell lines <5% blasts No Auer rods
	Pancytopenia with SLD	Pancytopenia No or rare blasts (<1%) No Auer rods	Dysplasia in $\geq 10\%$ of cells in one myeloid cell line only May have no or increased ring sideroblasts <5% blasts No Auer rods
	Absence of dysplasia with defining cytogenetics	Uni-, bi-, or pancytopenia No or rare blasts (<1%) No Auer rods	No significant morphological dysplasia, but requires an MDS-defining cytogenetic abnormality

*Denotes that $\geq 5\%$ ring sideroblasts qualifies if an *SF3B1* mutation is known.

or thrombocytopenia. Isolated neutropenia or thrombocytopenia is rare, and thorough investigation for alternate etiologies should be pursued as these are more common. The dysplastic lineage may or may not correspond to the lineage with cytopenias. To distinguish this from MDS with ring sideroblasts and single lineage dysplasia (MDS-RS-SLD), iron stains must be performed to exclude a significant increase in ring sideroblasts (<15% in patients without *SF3B1* mutations or <5% in patients with *SF3B1* mutations).

If blasts are 1% in the peripheral blood on two consecutive examinations, this signifies a worse prognosis than is typically

associated with MDS-SLD and should be classified as MDS, unclassified (MDS-U) as discussed later in this chapter. To distinguish this from MDS with excess blasts (MDS-EB), blasts should be quantified as <1% in the peripheral blood, <5% in the bone marrow, and no blasts with Auer rods should be present.

In the absence of hallmark clonal cytogenetic abnormalities, these cases may be difficult to distinguish from reactive or nonneoplastic causes of dysplasia or CCUS. As always, other etiologies of cytopenias or dysplasia should be excluded. Even if somatic mutations are present, dysplasia should be

present in at least 10% of the lineage to qualify as MDS-SLD, distinguishing these cases from CCUS. If a clonal cytogenetic abnormality is not present, observation is recommended for at least 6 months for the persistence of cytopenias and dysplasia before making a definitive diagnosis of MDS-SLD.

Cytogenetic findings are not specific, but MDS-SLD cases typically have good or very good cytogenetics in the IPSS-R scoring system. Overall, these cases tend to have a good prognosis.

MDS With Multilineage Dysplasia

MDS with multilineage dysplasia (MDS-MLD) cases show dysplasia in two or three lineages and may show cytopenias in one to three lineages in the peripheral blood. As with MDS-SLD, MDS-RS and MDS-EB should be excluded with iron stains showing no significant increase in ring sideroblasts, and peripheral blood and bone marrow showing no significant increase in blasts. Again, if blasts are 1% in the peripheral blood on two consecutive examinations, this signifies a worse prognosis than is typically associated with MDS-MLD, and should be classified as MDS, unclassified (MDS-U) as discussed later in this chapter.

Cytogenetic findings are not specific. These cases show more variability in cytogenetics and prognosis, with most cases qualifying as low or intermediate risk by the IPSS. Of note, patients with complex karyotypes have a worse prognosis, similar to those of patients with MDS-EB.

MDS With Ring Sideroblasts

MDS with ring sideroblasts (MDS-RS) cases fall under the classification of MDS-RS when they show either at least 15% ring sideroblasts in patients without *SF3B1* mutations or at least 5% ring sideroblasts in patients with *SF3B1* mutations. These cases can be further stratified into MDS-RS-SLD or MDS-RS-MLD depending on how many lineages show cytopenias in the peripheral blood and morphological dysplasia. Most patients present with anemia with or without neutropenia or thrombocytopenia. The peripheral blood in these cases may show a dimorphic population of red blood cells with most of the cells being normochromic and a subset showing hypochromia.

These cases should show <1% blasts in peripheral blood and <5% blasts in the bone marrow. If there are features of increased blasts (at least 2% in the peripheral blood or 5% in the bone marrow or the presence of Auer rods), they are better classified as MDS-EB.

Cytogenetic findings are nonspecific. *SF3B1* mutations are strongly associated with the phenotype of ring sideroblasts and are identified in anywhere from 30% to 90% of cases of MDS-SLD and MDS-MLD, with a higher frequency in MDS-SLD. Mutations in other spliceosomal proteins such as *SRSF2*, *U2AF1*, and *ZRSR2* can be seen in a minor subset of these cases and are generally mutually exclusive with *SF3B1* mutations.

The prognosis in these cases is generally better in MDS-RS-SLD than in MDS-RS-MLD, with cytogenetic findings in MDS-RS-MLD being an important factor in overall prognosis.

MDS With Isolated del(5q)

The hallmark of this category of MDS is a del(5q) on karyotype with or without one other cytogenetic abnormality that cannot include del(7q) or monosomy 7 (Figs. 20–27 to 20–28). These cases show one or two cytopenias, typically anemia with or

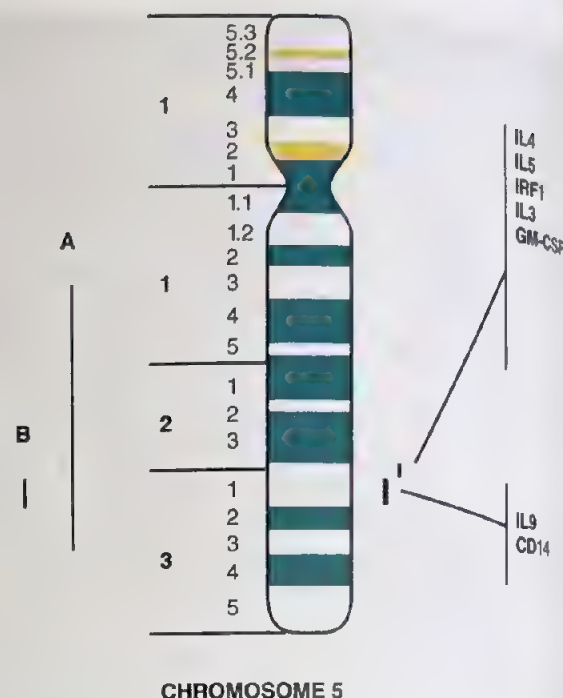


FIGURE 20-27 MDS with isolated del 5q: Several genes related to hemopoiesis are localized on 5q. A. The interstitial deletion may comprise any region located between bands 5q13 and 5q33. B. The band 5q31 is consistently deleted in most patients.

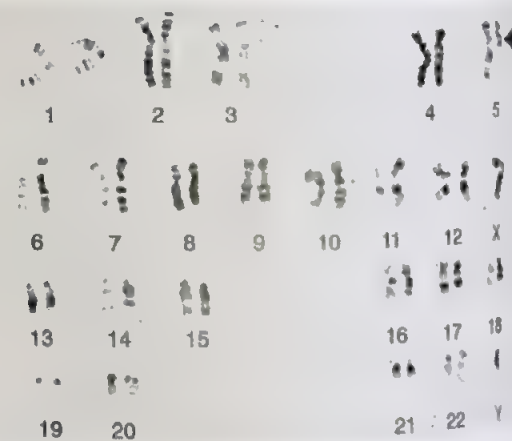


FIGURE 20-28 Karyotype (GTG banding), showing the interstitial deletion of chromosome 5 → 46,XY,del(5)(q13q33) characteristically found in MDS with isolated del 5q.

without another cytopenia, and dysplasia in one to three lineages. Pancytopenia should not be present. Ring sideroblasts may or may not be increased, and blasts should not be increased. Cases may show Auer rods (<1% in peripheral blood and <5% in bone marrow). MDS with isolated del(5q) is a low-risk category with a better prognosis than other categories. Unique features of this MDS subtype are a predominance in women rather than men as is seen in most other subtypes of MDS, and the presence of thrombocytosis in up to 50% of cases.

MDS With Excess Blasts

MDS with excess blasts (MDS-EB) is diagnosed when blasts are increased in peripheral blood or bone marrow and can be further subclassified into MDS-EB-1 and MDS-EB-2 based on the degree of increased blasts and the presence of blasts

with Auer rods. MDS-EB-1 shows 2% to 4% blasts in the peripheral blood or 5% to 9% blasts in the bone marrow, and no blasts with Auer rods, whereas MDS-EB-2 shows 5% to 10% blasts in the peripheral blood or 10% to 19% blasts in the bone marrow, or any blasts with Auer rods. This strategy highlights the importance of morphological evaluation of the blasts for the presence of Auer rods, as this categorizes the disease as higher risk. MDS-EB can show cytopenias in one or two lineages and dysplasia in one to three lineages and can be diagnosed with any cytogenetic findings. Dysplasia in all three lineages is frequent. Patients with MDS-EB have a worse prognosis with higher rates of progression to AML regardless of cytogenetic or somatic mutations present.

Cases of MDS with significant reticulin fibrosis, termed MDS-F, tend to fall into this category with excess blasts. The presence of significant fibrosis confers a worse prognosis regardless of blast count, but it is important to note that fibrosis may complicate blast percentage estimation by contributing to inadequate aspirates.

MDS, Unclassified

MDS unclassified (MDS-U) should not be considered a "wastebasket" category when other categories don't fit but should be diagnosed in three specific situations: when peripheral blood blasts are persistently 1% but blast counts in the marrow are otherwise low; when there are trilineage cytopenias but only unilineage dysplasia; and when there is no significant dysplasia but a MDS-defining cytogenetic abnormality is present.

As noted previously, cases that would otherwise classify as MDS-SLD, MDS-MLD, MDS-RS, or MDS with isolated del(5q) with 1% peripheral blood blasts documented on two consecutive evaluations have a worse prognosis than is typical for those entities. Cases that would otherwise suggest MDS-SLD, MDS-RS-SLD, or MDS with isolated del(5q) that present with pancytopenia are also classified as MDS-U. It is important to note that the use of the definition of cytopenia provided by IPSS-R, which differs from hospital laboratories reference ranges, should be used to define cytopenias and thereby prevent overdiagnosis of MDS-U. These are hemoglobin <10 g/dL, platelet count <100 K/uL, and ANC <1.8 K/uL. In cases with persistent cytopenias, no significant increase in blasts (<2% in peripheral blood and <5% in bone marrow), no significant dysplasia, and the presence of an MDS-defining cytogenetic abnormality should also be diagnosed as MDS-U. The prognosis of these cases is less certain; monitoring for any features that would allow specific classification is recommended.

This chapter was completed prior to the publication of the 2022 edition of the World Health Organization classification of myelodysplastic neoplasms. Table 20-4 compares the WHO revised 4th edition classification with WHO 5th edition classification.

TABLE 20-4 Comparison of the WHO Revised Fourth Edition With WHO Fifth Edition Classification

WHO Classification, Revised Fourth Edition 2016	WHO Classification, Fifth Edition 2022
Myelodysplastic Syndromes (MDSs)	Myelodysplastic Neoplasms (MDNs)
MDS with single lineage dysplasia	MDN, with defining genetic abnormalities
MDS with ring sideroblasts	MDN with low blasts and 5q deletion
MDS with multilineage dysplasia	MDN with low blasts and SF3B1 mutation
MDS with excess blasts	MDN with biallelic TP53 inactivation
MDS with excess blasts and erythroid predominance	MDN, morphologically defined
MDS with excess blasts and fibrosis	MDN with low blasts
	MDN, hypoplastic
	MDN with increased blasts
MDS with isolated del(5q)	
MDS, unclassifiable	
Childhood MDS	MDNs of childhood
Refractory cytopenia of childhood	Childhood MDN with low blasts
	Childhood MDN with increased blasts

Adapted from: Zhang, Y., Wu, J., Qin, T., Xu Z., Qu S., Pan L., et al. Comparison of the revised 4th (2016) and 5th (2022) editions of the World Health Organization classification of myelodysplastic neoplasms. *Leukemia* 36, 2875–2882 (2022).

may be present²⁸ (Fig. 20-29). Other infiltrates that may not aspirate well can also be seen on the core biopsy. It is well accepted that the most accurate estimate of bone marrow cellularity is made from an adequate biopsy specimen. Bone marrow biopsy is especially relevant in cases with significant bone marrow fibrosis, where paucicellular aspirate smears are common and may lead to difficult

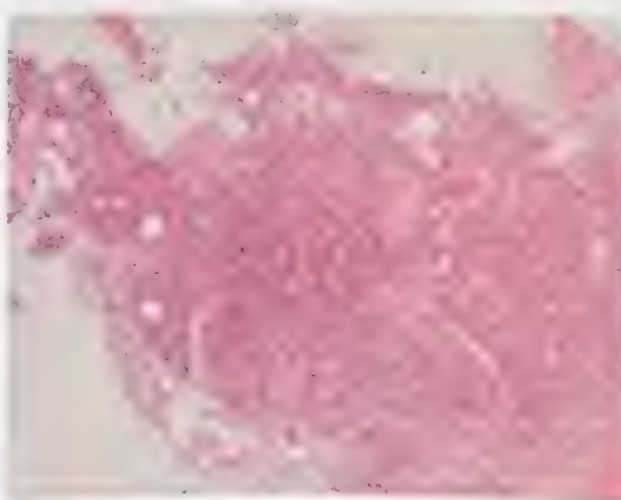


FIGURE 20-29 Hypercellularity (bone marrow core): the cellularity here is estimated to be >95% with few adipose cells remaining.

Laboratory Testing and Results

Bone Marrow Histology

Assessment of bone marrow core biopsies is important for determining bone marrow cellularity, erythroid or myeloid predominance, and any fibrosis or stromal changes that

estimation of blast percentage (Fig. 20-30). In addition to the assessment of cellularity and blast percentages, the bone marrow biopsy specimen is helpful in revealing the disruption of normal hematopoietic architecture, with displacement of granulopoiesis, erythropoiesis, and megakaryopoiesis from their usual sites. Abnormal localization of immature precursors (ALIP), clusters or aggregates of myeloblasts, and promyelocytes of usually three or more cells distant from the bone marrow trabeculae are also features of some MDS²³ (Fig. 20-31). Although dyserythropoiesis and dysgranulopoiesis are difficult to assess on bone marrow biopsy specimens, dysmegakaryopoiesis can be fairly easily estimated.

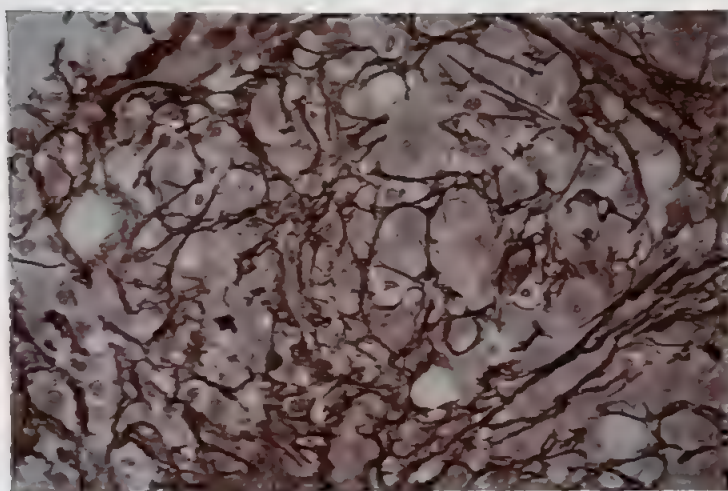


FIGURE 20-30 MDS with severe fibrosis (bone marrow core, reticulin stain): severe fibrosis highlighted by dense, intersecting bundles of reticulin fibers.

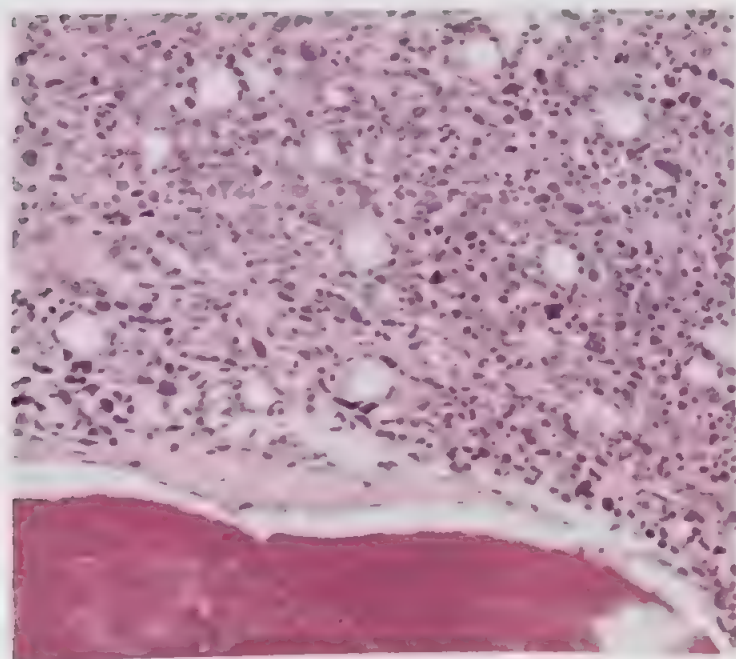


FIGURE 20-31 Abnormal localization of immature precursors (ALIP) (bone marrow core): increased immature cells with larger nuclei and pale chromatin are shown in aggregates to sheets located away from the bone trabeculae.

ADVANCED CONTENT

When there are discrepancies in the blast percentage determined by the bone marrow aspirate count and the estimation of immature appearing cells in the core biopsy, immunohistochemical stains can help determine the accurate blast count necessary for proper classification (Figs. 20-32 to 20-33). CD34 is often used as a marker of myeloblasts, but other stains such as CD117 may be used, especially when the blasts may be aberrantly CD34 negative. CD117 does stain for other early precursors (pronormoblasts, promyelocytes, etc.), which is important to consider when using it to identify blasts.

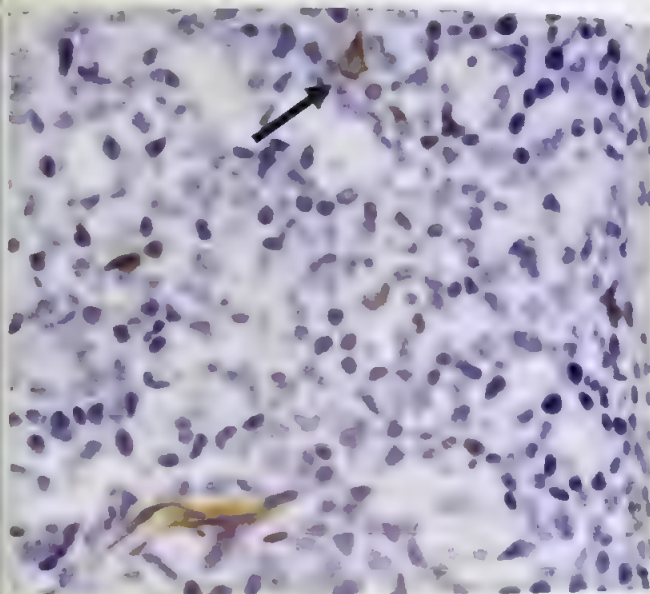


FIGURE 20-32 Blast estimate by CD34 (bone marrow core, CD34 stain): CD34 stains vessels, seen at the lower left as a longitudinal structure, as well as rare blasts (arrow).

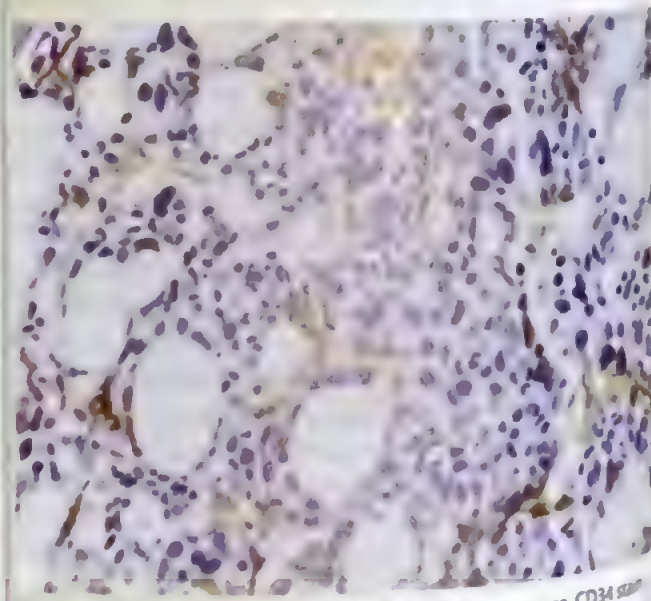


FIGURE 20-33 Blast estimate by CD34 (bone marrow core, CD34 stain): here the blasts are increased, estimated at 10%-15% by CD34 immunohistochemical stain.

ADVANCED CONTENT

Flow cytometric findings are not incorporated into the diagnostic criteria or prognostic categories of MDS, but clues to the diagnosis may be identified by flow cytometry. Blast enumeration by flow cytometry can suggest the presence of increased myeloblasts; however, morphological determination of blast percentage is the gold standard and should be used for classification. This may be partially due to hemodilution of flow cytometry specimens.²⁹ Phenotypic aberrancies may be seen on myeloblasts, such as the expression of B-cell or T-cell lineage markers. The normal immature B-cell compartment, hematogones, may be decreased relative to myeloblasts. Maturing granulocytes may show decreased side scatter, reflecting the hypogranularity observed on morphology.²⁹ Several flow cytometry scoring indexes have been proposed incorporating these findings and others to predict the presence of MDS; however, their utility in the diagnosis of MDS is still unclear.²⁹

Cytogenetic and Molecular Abnormalities

A full description of the terminology and abbreviations used in describing chromosomes and their abnormalities is beyond the scope of this chapter. For detailed guidelines, the reader is referred to a specialty textbook. A brief review adapted for the comprehension of this section is presented in Table 20-5.

Since the introduction of the FAB classification of MDS in 1982, cytogenetics has become one of the most informative

laboratory tools in their investigation and management. The nature and complexity of clonal chromosomal abnormalities have proven to be essential in diagnosis and prognosis. As advances in high-throughput sequencing technologies have been made, our understanding of the importance of single gene mutations in the pathogenesis, behavior, and prognosis of MDS have also progressed. As noted previously, chromosomal and molecular aberrancies are a key to establishing the clonal etiology of MDS. Specific abnormalities can contribute independent prognostic factors, and thus provide the opportunity to individualize therapy.

Clonal cytogenetic abnormalities are detected in about 50% of de novo MDS cases and the incidence rises to close to 90% in t-MDS.^{9,30} Structural and numerical anomalies are found, but chromosomal deletions are by far the most frequent and characteristic cytogenetic events observed. The most frequent anomalies include partial deletions of chromosome arms 5q, 20q, or 7q; loss of chromosome 7 or chromosome Y; trisomy 8, and anomalies of the short arm of chromosome 17¹ (Fig. 20-34). The cytogenetic anomalies reported in t-MDS are related to the type of therapy that patients have previously received. Approximately 70% of patients treated with an alkylating agent show anomalies of chromosome 5 or 7, or both, whereas abnormalities of 11q23 are more frequently seen in patients with exposure to topoisomerase II inhibitors, such as doxorubicin and etoposide.^{1,30} Complex karyotypes (three or more chromosomal abnormalities within the same cell) are found in about 20% of de novo MDS and in 40% to 90% of t-MDS.³⁰

As our understanding of the underlying cytogenetics of MDS has evolved, a presumptive diagnosis of MDS can now be made in patients with cytopenias and certain suggestive cytogenetic findings, even in the absence of morphological dysplasia.

Cytogenetic analysis provides an important prognostic tool for the risk of transformation to AML and for survival, as seen incorporated in the IPSS-R classification (see Table 20-1). Patients with monosomy 7, chromosome 3 abnormalities, or complex karyotypes have a short survival and a high rate of progression to AML. Patients with -Y or del 11q as a sole

TABLE 20-5 Abbreviations and Terminology Used in Describing Chromosomes and Their Abnormalities

Abbreviation	Description
p	Short arm of a chromosome
q	Long arm of a chromosome
q-	Loss of chromosome material to long arm
del	Deletion of chromosome material
t	Translocation of chromosome material (DNA exchange between 2 chromosomes)
mar	Marker chromosome that is not fully characterized
+	Addition of a chromosome
-	Loss of a chromosome
hypodiploid	Cells having fewer than 46 chromosomes
hyperdiploid	Cells having more than 46 chromosomes
Complex karyotype	The presence of 3 or more chromosomal abnormalities in the same cell

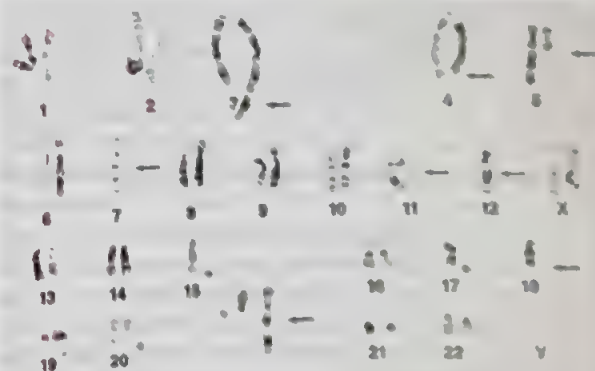


FIGURE 20-34 Karyotype (GTG banding), showing complex clonal abnormalities involving at least 8 chromosomes within the same cell (a) including del(5)(q13q33), -7, del(20)(q11.2)

anomaly have a more favorable clinical outcome, with a tendency for a long leukemia-free survival.

Molecular studies showing genetic mutations can also be used to establish clonality in MDS. Although molecular aberrancies alone cannot confer a diagnosis of MDS without the correct clinical and morphological setting (see diagnostic pitfalls section on CHIP and CCUS), particular single gene mutations are important for proper classification and prognosis, such as the relatively good prognosis of *SF3B1* mutations in patients with ring sideroblasts.

CRITICAL THINKING QUESTION

20-2 Why would a bone marrow biopsy need to be ordered in addition to a bone marrow aspirate for MDS diagnosis?

Therapy-Related Myelodysplastic Syndromes

MDS is said to be secondary, or therapy-related, when it occurs after significant exposure to chemotherapy or radiotherapy. The laboratory findings, clinical manifestations, and evolution resemble those of de novo MDS, but t-MDS show a higher frequency of chromosomal abnormalities and a greater tendency to early leukemic transformation, particularly if a complex karyotype is present. The prognosis is generally poorer in t-MDS than in de novo MDS.

It should be noted that t-MDS are not categorized with de novo MDS under the 2017 WHO classification, but rather under the umbrella category of therapy-related myeloid neoplasms, which importantly does not make a distinction between t-MDS and t-AML. In this scheme, the prognostic value is in the cytogenetic profile. Thus, the blast count that would normally stratify a case into MDS versus AML is largely irrelevant.

ADVANCED CONTENT

There has been an increase in the number of patients diagnosed with t-MDS in recent years, which is likely attributable to the increased usage of intensive chemotherapy protocols and greater survivorship among those treated for other cancers. The risk of t-MDS following chemotherapy and/or radiation therapy is variable depending on the treatment regimen used but is roughly 5-fold over the general population.³¹ Most cases are causally related to previous therapy with alkylating agents, almost all of which have been shown to be leukemogenic because of direct DNA damage. The period of latency between exposure to alkylating agents and development of t-MDS is usually 4 to 7 years, and patients usually have a poor response to therapy with the exception of allogeneic bone marrow transplantation.³¹ In patients with MDS and a history of previous

exposure to alkylating agents, cytogenetic studies usually show unbalanced cytogenetic aberrations, primarily -7 , -5 , $5q-$, and $7q-$.

Topoisomerase II inhibitors are associated with t-MDS with a shorter latency period of 1 to 5 years and are more likely to present as a therapy-related AML without a preceding t-MDS.³¹ DNA topoisomerases are a class of enzymes important in various DNA transactions such as replication, transcription, and recombination. Most of the balanced translocations involve 11q23 and different partners such as chromosomes 9 or 19.³¹ Usually the *KMT2A* (previously *MLL*) gene (located at 11q23) is rearranged. Other balanced translocations seen as recurrent cytogenetic abnormalities in AML such as t(8;21) have also been reported in this setting but are rare.³¹ The t-MDS caused by topoisomerase II inhibitors responds better to treatment than that caused by alkylating agents.

Myelodysplastic Syndromes in Children

MDS occurs rarely in children, and most cases are classified in the WHO provisionally as refractory cytopenia of childhood (RCC). Children are most often symptomatic with fever, malaise, infection, or bleeding.¹ Unlike adults who most often present with isolated anemia, children more often present with thrombocytopenia and neutropenia, in addition to anemia in approximately 50%.^{1,32} Cases of MDS in children can be associated with predisposing bone marrow failure syndromes, such as Fanconi Anemia; germline predisposition syndromes, such as inherited mutations in *RUNX1*; and with previous predisposing therapies. A challenge in pediatric MDS is frequent hypocellular bone marrows, making the distinction from aplastic anemia and bone marrow failure syndromes more challenging.³² Thorough clinical assessment for syndromic findings and family history as well as laboratory assessment to exclude bone marrow failure syndromes is necessary.³²

Diagnostic Challenge

Some MDS patients carry features shared with other hematologic disorders. These clinical situations may lead to confusion concerning the appropriate diagnosis for the patient. The most common of these difficult diagnostic quandaries are presented next.

Reactive Causes of Dysplasia

Many benign conditions can be associated with both peripheral blood cytopenias and morphological dysplasia, including chronic infections (especially viral), autoimmune disorders, medications, alcohol and other toxins, and nutritional deficiencies^{1,23} (see Box 20-2). A clinical history suggestive of one of these disorders and an accurate medication list are essential to tipping off the pathologist to the likelihood of an alternate diagnosis to MDS. Morphological findings may also be present, which could suggest a specific alternate diagnosis such as vacuolization of erythroid and myeloid precursors in conjunction with ring sideroblasts in copper deficiency (Figs. 20-35 to 20-36).

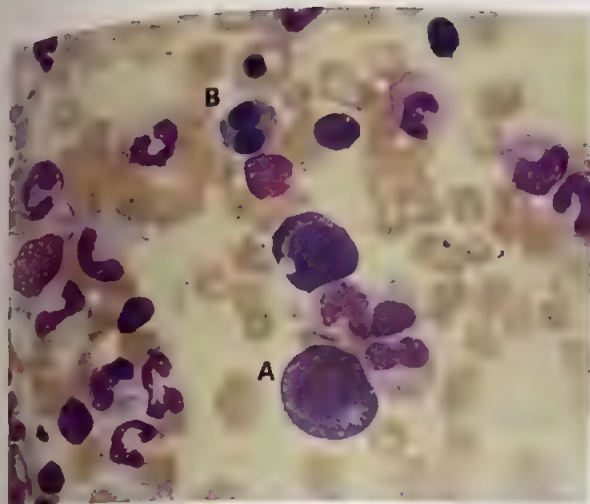


FIGURE 20-35 Copper deficiency (bone marrow aspirate): vacuolization of erythroid precursor (A) is seen with a binucleate form (B); in this case the dysplasia is caused by copper deficiency rather than a neoplastic disorder.

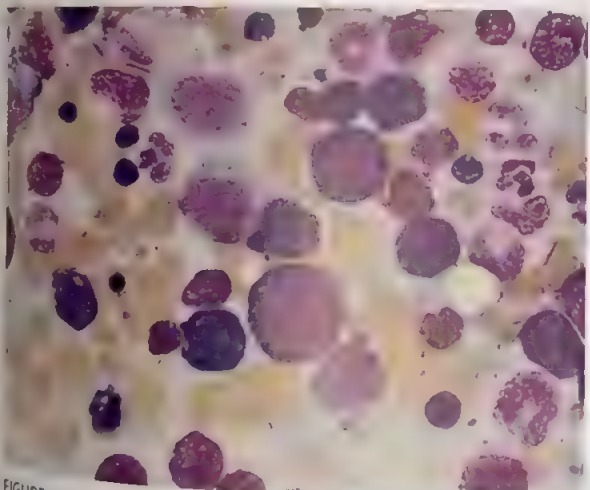


FIGURE 20-36 Megaloblastic anemia (bone marrow aspirate): erythroid hyperplasia with increased immature forms is seen with abnormal nuclear contours in this case of megaloblastic anemia secondary to vitamin deficiency.

Cytogenetic and molecular findings may be helpful to confirm the presence of clonal hematopoiesis. Although many cases of true MDS may have a normal karyotype, the high negative predictive value of NGS myeloid panels can help exclude MDS when nonneoplastic diseases are being considered.

Cytogenetic and Molecular Findings Without Morphological Dysplasia

Evidence of clonal hematopoiesis such as cytogenetic and molecular aberrancies must be taken in the context of cytopenias and bone marrow morphological findings for proper interpretation. In patients with persistent cytopenias undergoing evaluation for MDS, some cytogenetic and molecular aberrancies may be sufficient for a presumptive diagnosis of MDS even in the absence of morphological dysplasia, but the

significance of other cytogenetic and molecular abnormalities is uncertain.

In patients with cytopenias and certain genetic findings typical of MDS, a presumptive diagnosis of MDS can be made. Other cytogenetic findings may be of uncertain significance, such as loss of chromosome Y, which, especially when present in only a subset of cells evaluated, may be considered a normal age-related finding.³³ Patients with cytopenias and a few myeloid associated somatic mutations, especially at low mutational burden (or variant allele fraction), may be best classified as CCUS. The risk of underlying myeloid neoplasia or progression to frank MDS depends on the number of somatic mutations, the pattern of mutations, and the mutational burden.⁷ In these patients, serial monitoring for progression of cytopenias or development of clinical symptoms is helpful to identify those with progression to a myeloid neoplasm.²

MDS With Hypoplastic Marrow

Approximately 10% to 15% of MDS patients have a hypocellular bone marrow.²³ It may be difficult to differentiate such cases from aplastic anemia. This is especially complex given that aplastic anemia may progress to and probably has some biological overlap with MDS. The presence of increased blasts and/or significant morphological dysplasia favor MDS over aplastic anemia. There is some controversy over whether any karyotypic abnormality excludes the possibility of aplastic anemia, but typical cytogenetic and molecular features of MDS would favor a hypoplastic MDS.²³ In some cases, it may not be possible to differentiate between the two.

Treatment

Currently, **allogeneic stem cell transplantation**, where the bone marrow stem cells from another individual with normal hematopoiesis are transplanted into the patient, is the only therapeutic modality with the potential to cure MDS. However, as most patients with MDS are elderly, they are not amenable to transplantation and are therefore incurable. As there is significant prognostic heterogeneity among MDS patients, the IPSS-R and other proposed prognostic classifications are invaluable tools that help tailor therapy to the predicted prognosis.²⁰ Broadly, therapies can be organized into supportive care with those directed at palliation of symptomatic cytopenias versus those directed at the disease itself.²⁰

Supportive Care and Hematopoiesis-Improving Therapies

For elderly patients with good prognosis MDS, the mainstay of treatment consists of supportive measures. For patients who are asymptomatic with mild cytopenias and have low-risk MDS, observation may be appropriate.³⁴ Transfusion of packed red blood cells may alleviate symptoms related to anemia. Chelation therapy with desferoxamine or novel oral chelators may be indicated in some heavily transfused patients to prevent secondary hemochromatosis.^{18,34} Platelets can also be transfused to patients who bleed.

ADVANCED CONTENT

The use of growth factors has been widely studied in MDS. Erythroid-stimulating agents have been used in MDS. These agents have shown improvement in transfusion dependence without clear survival benefit.¹⁸ Similarly, thrombopoietin agonists to stimulate platelet production have been used in the context of clinical trials.¹⁸

Immunosuppressive therapy using antithymocyte globulin (ATG) and other anti-T-cell immunosuppressive agents has been used in a subset of patients.

The most significant pharmacological development for low-grade MDS patients has been the introduction of lenalidomide. Lenalidomide is a structural analogue of thalidomide that lacks teratogenicity and most neurological side effects of the parent drug. The exact mechanism of action of lenalidomide is not fully characterized, but the molecule exhibits immunoregulatory and antiangiogenic effects, which are likely to contribute to its clinical efficacy. This has become the standard of care in a subset of low-risk patients with del5q, again emphasizing the importance of cytogenetics in therapy selection.^{18,34}

Therapies Oriented Toward Improving Survival

Patients with advanced MDS should be considered for allogeneic stem cell transplantation (alloSCT), the only curative treatment available for MDS. However, stem cell transplantation is usually limited to younger patients who have a suitable donor.¹⁸ In addition, whether there is a benefit for alloSCT in patients who have otherwise achieved a response with hypomethylating therapies is unclear.¹⁸

ADVANCED CONTENT

High-dose chemotherapy (without transplantation) with regimens similar to those used to induce remission in acute leukemia has met with disappointing results. Although a hematological response can be achieved, response rates are lower than those observed in AML. In addition, relatively few patients are candidates for intensive AML-like therapy due to the elderly population.¹⁸

Targeting genes that have become hypermethylated (and thus underexpressed) during disease progression with hypomethylating agents such as 5-azacytidine (Vidaza) or decitabine (Dacogen) has been associated with hematologic response, including transfusion independence often observed. In high-risk MDS, these hypomethylating therapies have become the standard of care and have also shown a delayed progression to AML and improved quality of life.^{18,34}

Recent advances in the understanding of the pathogenesis of MDS have paved the way to the development of novel drugs that may prove promising to improve the quality of life and potentially the survival of MDS patients, expanding therapeutic options for MDS and generating new hope for patients.

Myelodysplastic/Myeloproliferative Overlap Syndromes

Myelodysplastic/myeloproliferative overlap syndromes are characterized by overlapping features of myelodysplastic syndromes, frequently exhibiting dysplasia, and peripheral blood cytoeses characteristic of myeloproliferative neoplasms instead of cytopenias. These overlap syndromes were previously categorized as MDS in the first FAB classification, but they now constitute a separate group of entities in the WHO classification system. The 2016 WHO classification categories of the myelodysplastic/myeloproliferative diseases are:

- Chronic myelomonocytic leukemia
- Atypical chronic myeloid leukemia, *BCR-ABL1*-negative
- Juvenile myelomonocytic leukemia
- Myelodysplastic/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis
- Myelodysplastic/myeloproliferative neoplasm, unclassifiable

An example with diagnostic criteria for chronic myelomonocytic leukemia (CMML), the most common MDS/MPN neoplasm, is presented in Box 20-3. Examples of the morphological findings with peripheral blood monocytois and bone marrow promonocytes are seen in Figures 20-37 and 20-38.

Similar to MDS, MDS/MPN overlap syndromes frequently exhibit cytogenetic and molecular abnormalities, but no single

BOX 20-3 2016 WHO Diagnostic Criteria for Chronic Myelomonocytic Leukemia (CMML)

- Persistent peripheral blood monocytois greater than $1 \times 10^9/L$ with monocytes accounting for $\geq 10\%$ of the leukocytes
- WHO criteria for *BCR-ABL1*-positive chronic myeloid leukemia, primary myelofibrosis, polycythemia vera, and essential thrombocythemia are not met
- No rearrangement of *PDGFRA*, *PDGFRB*, or *FGFR1* and no *PCM1-JAK2* (which should be specifically excluded in cases with eosinophilia)
- Fewer than 20% blasts* in the blood and bone marrow
- Dysplasia in one or more myeloid lineages. If myelodysplasia is absent or minimal, the diagnosis of CMML may still be made if the other requirements are present and an acquired, clonal cytogenetic abnormality is present in the hematopoietic cells or the monocytois has been persistent for at least 3 months and all other causes of monocytois have been excluded.
- Diagnose CMML-0 when blasts are less than 2% in the blood and less than 5% in the bone marrow, and no Auer rods are present
- Diagnose CMML-1 when there are 2% to 4% blasts in blood and 5-9% in bone marrow, and no Auer rods are present
- Diagnose CMML-2 when blasts are 5% to 19% in blood or 10% to 19% in marrow, or if Auer rods are present and blasts are fewer than 20% in blood or marrow

*In this classification of CMML, blasts include myeloblasts, monoblasts, and promonocytes.

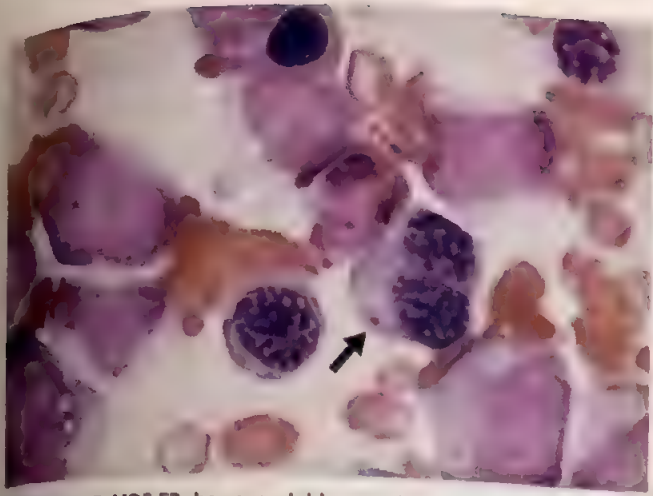


FIGURE 20-37 MDS-EB shows myeloblasts and dyserythropoiesis. Note the Howell-Jolly body (arrow).

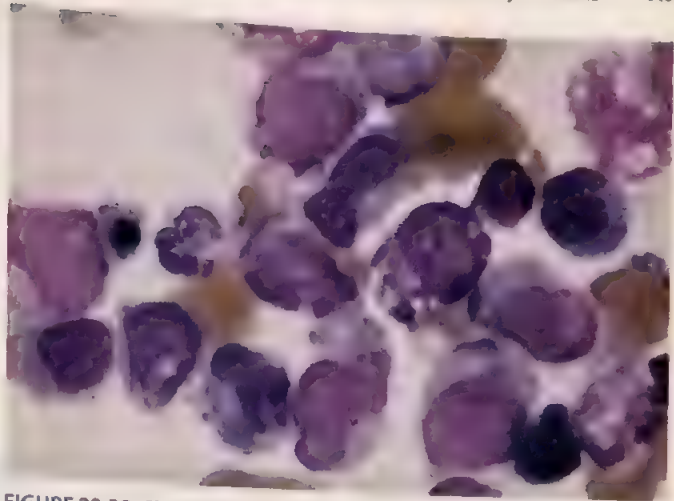


FIGURE 20-38 Chronic myelomonocytic leukemia (CMML): myeloblasts and promonocytes are seen with increased monocytes in the bone marrow. (Bone marrow aspirate).

diagnostic, defining abnormality is present. The presence of mutations typically associated with myeloproliferative neoplasms such as *JAK2*, *CALR*, or *MPL* can indicate a myeloproliferative component. These syndromes are heterogeneous with varying characteristics, prognosis, and therapeutic strategies.

Similar therapies as used in MDS can be used in some MDS/MPN overlap syndromes, including hypomethylating agents and, ultimately, alloSCT if clinically feasible. In other cases, treatment modalities resemble those used in myeloproliferative neoplasms, such as hydroxyurea.^{35,36}

SUMMARY CHART

- Myelodysplastic syndromes (MDS) are clonal hematologic malignancies characterized by peripheral blood cytopenias, dysplastic cells in peripheral blood and bone marrow, and a propensity to transform into acute leukemia.
- Diagnosis of MDS is based on a combination of clinical history, laboratory findings, and morphological evaluation of blood and bone marrow smears, and often requires ancillary testing for definitive diagnosis and classification.
- MDS is characterized by morphological abnormalities which may include dyserythropoiesis, dysgranulopoiesis, and dysmegakaryopoiesis.
- Several factors influence the prognosis in MDS. The most significant are depth of cytopenias, cytogenetic abnormalities, and bone marrow blast percentage.
- The Revised International Prognosis Scoring System (IPSS-R) helps determine the prognosis of individuals with MDS.
- Anemia is the most common presenting cytopenia in adults and is often macrocytic or normocytic with decreased reticulocyte count.
- Peripheral blood findings that raise suspicion for MDS include macrocytic anemia with anisopoikilocytosis, granulocytes with abnormal nuclear segmentation and hypogranulation, and large hypogranular platelets.
- Bone marrow aspirate smears may show dyserythropoiesis with a wide variety of nuclear lobation abnormalities and nuclear to cytoplasmic dyssynchrony. Assessment of iron stains on the aspirate smears is important for the identification of ring sideroblasts. Dysmegakaryopoiesis is frequently characterized by small hypolobate forms and occasionally forms with separated nuclear lobes.
- The 2016 WHO classification of MDS consists of six subtypes: MDS with single lineage dysplasia (MDS-SLD), MDS with multilineage dysplasia (MDS-MLD), MDS with ring sideroblasts (MDS-RS), MDS with isolated del(5q), MDS with excess blasts (MDS-EB), and MDS, unclassifiable (MDS-U).
- Bone marrow biopsy is helpful to assess cellularity, erythroid or myeloid predominance, morphological dysplasia, abnormal localization of immature precursors (ALIP), and myelofibrosis.
- Clonal cytogenetic abnormalities are detected in approximately 50% of patients with de novo MDS. The most frequent abnormalities are del 5q, monosomy 7 or del 7q, trisomy 8, and deletion of part of chromosome 20.
- Clonal somatic mutations are found in 10% of elderly patients with no known diagnosis but are also found, typically in more genes and with higher mutational burdens, in patients with MDS.

SUMMARY CHART—cont'd

- Secondary myelodysplastic syndrome is often therapy related (t-MDS) and occurs after significant exposure to chemotherapy or radiation therapy. The laboratory findings, clinical manifestations, and evolution resemble those of de novo MDS, except for a higher frequency of chromosomal abnormalities and worse prognosis.
- MDS occurs rarely in children but is associated with inherited bone marrow failure syndromes, germline predisposition syndromes, or prior therapy.
- At the present time, allogeneic hematopoietic stem cell transplantation is the only curative treatment for MDS.

CASE STUDY 20-1

A 72-year-old man was admitted to the hospital with complaints of increasing weakness. Physical examination revealed pallor but no organomegaly. His previous medical history was unremarkable.

The laboratory results were as follows:

CBC

WBC	$2.9 \times 10^9/L$
RBC	$2.43 \times 10^{12}/L$
Hgb	9.1 g/dL
Hct	27%
MCV	111 fL
MCH	37.4 pg
MCHC	33.7 g/dL
RDW	19.0%
PLT	$90 \times 10^9/L$
MPV	17.4 fL

Blood Smear Differential and Morphology

Blasts	2%
Promyelocytes	0%
Myelocytes	1%
Metamyelocytes	0%
Segmented neutrophils	72%
Eosinophils	1%
Basophils	1%
Lymphocytes	20%
Monocytes	3%

Additional findings at the blood smear: occasional pseudo-Pelger-Huët neutrophils with hypogranulation, occasional giant platelets, erythrocyte anisomacrocytosis with occasional basophilic stippling.

BONE MARROW ASPIRATE

Blasts: 15%, a few blasts with Auer rods

Dysgranulopoiesis with immature cells with asynchronous nuclear-cytoplasmic maturation

Pseudo-Pelger-Huet cells with hypogranulation

Left shifted erythroid maturation with megaloblastoid changes

Micromegakaryocytes with nuclear hypolobation

Prussian blue stain showed 10% ring sideroblasts

CYTOGENETICS

Complex karyotype, including monosomy 7

QUESTIONS:

1. Are there any cytopenias present? If so, which?
2. What does the peripheral smear reveal about this patient?
3. Based on the bone marrow aspirate, which subtype of MDS does this patient most likely fall within?

ANSWERS:

1. Macrocytic anemia and thrombocytopenia.
2. There are blasts present, along with pseudo-Pelger-Huet cells with hypogranulation, anisocytosis, basophilic stippling, and some giant platelets. All of these findings are consistent with MDS.
3. With 15% blasts and the presence of Auer rods, this is likely MDS-EB-2.

CASE STUDY 20-2

A 32-year-old male has a history of Hodgkin lymphoma diagnosed in his early 20s, treated with chemotherapy and now in remission for 10 years, and presents for routine care.

CBC	
WBC	$3.1 \times 10^9/L$
RBC	$3.56 \times 10^{12}/L$
Hgb	11.7 g/dL
Hct	31%
MCV	105 fL
MCH	33.6 pg
MCHC	32.5 g/dL
RDW	17.5%
PLT	$135 \times 10^9/L$
MPV	10.9 fL

Blood Smear Differential and Morphology

Blasts	2%
Promyelocytes	0%
Myelocytes	0%
Metamyelocytes	0%
Segmented neutrophils	40%
Eosinophils	2%
Basophils	1%
Lymphocytes	49%
Monocytes	6%

Additional findings on the blood smear: Macrocytic red blood cells with anisopoikilocytosis, a few dysplastic

appearing neutrophils with cytoplasmic hypogranularity are present

BONE MARROW ASPIRATE

Blasts: 6%

Dysgranulopoiesis with >10% of the maturing granulocytes showing cytoplasmic hypogranularity

Erythroids show a spectrum of nuclear lobation abnormalities with >10% showing binucleation or irregular nuclear contours

Megakaryocytes appear increased with a few small, hypolobate forms (<10%)

Prussian Blue stain showed no increase in ring sideroblasts

CYTOGENETICS

Karyotype shows a complex karyotype with multiple chromosomal deletions, including del(5q) and del(17p)

QUESTIONS:

- Looking at the entire CBC and differential picture as well as the bone marrow aspirate, which findings align with MDS?
- What is most likely this patient's etiology for MDS?

ANSWERS:

- Neutropenia and the presence of blasts with hypogranulation.
- His history of being on chemotherapy drugs is likely causing t-MDS.

REVIEW QUESTIONS

- Which statement is true about clonal proliferation in MDS?
 - The mutation always impacts cellular maturation.
 - The mutations occur in younger patients more often than older patients.
 - Benzene is a known chemical agent that can induce mutations.
 - The mutations always affect cellular function.
- Fatigue, weakness, and dyspnea can all be attributed to which clinical finding in MDS?
 - Anemia
 - Thrombocytopenia
 - Neutropenia
 - Splenomegaly
- Prognosis is important for patients with MDS because of which of the following?
 - Risk of splenomegaly
 - Risk of transformation to CLL
 - Risk of infections
 - Risk of transformation to AML
- Dysplastic cells need to be above what threshold for diagnosis of MDS?
 - 5%
 - 10%
 - 25%
 - 50%
- What cellular inclusion is important in classification of MDS?
 - Auer rods
 - Hgb C crystals
 - Howell-Jolly bodies
 - Heinz bodies
- The presence of Auer rods results in a diagnosis of which MDS subtype?
 - MDS-SLD
 - MDS-RS-SLD
 - MDS-EB-1
 - MDS-EB-2

REVIEW QUESTIONS—cont'd

7. Recent increases in t-MDS is likely due to which?
 - a. Increased exposure to toxins
 - b. Increased use of chemotherapy drugs
 - c. Increased environmental toxins
 - d. More people living longer
8. Which of the following causes of cytopenia or cellular dysplasia resembles the cellular changes seen in MDS?
 - a. Chronic viral infections
 - b. Iron-deficiency anemia
 - c. Acute lymphoblastic leukemia
 - d. Bacterial infections
9. Which of the following is a curative therapy for MDS?
 - a. Blood transfusion
 - b. Oral chelators
 - c. Stem cell transplant
 - d. Platelet transfusion
10. The most common MDS/MPN disorder is which of the following?
 - a. AML
 - b. CLL
 - c. CML
 - d. CMML
11. Which erythroid finding is considered equivalent to erythroid dysplasia?
 - a. Howell–Jolly bodies
 - b. Heinz bodies
 - c. Poikilocytosis
 - d. Increased ringed sideroblasts
12. What is pseudo-Pelger–Huet anomaly?
 - a. Neutrophils with hypersegmentation
 - b. Agranular neutrophils
 - c. Hyposegmented neutrophils with hypogranulation
 - d. Neutrophils with toxic granulation
13. In addition to evaluation the bone marrow aspirate and peripheral blood for cytopenias and blasts, what other lab analysis can provide a diagnostic picture for MDS?
 - a. CBC
 - b. Cytogenetic analysis for recurrent anomalies
 - c. Immunoassays
 - d. Urinalysis

See answers at the back of this book.

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Chronic Lymphocytic Leukemia and Related Lymphoproliferative Disorders

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CHAPTER OUTLINE

Overview of Chronic Lymphocytic Leukemia
 Normal B Cell Development
 Classification of Lymphoid Neoplasms
 Hematologic Abnormalities
 Epidemiology
 Etiology
 Pathophysiology
 Phenotypic Features and Methods for Studying Lymphocytes
 Clinical Findings

Laboratory Testing and Results
 Genetic Abnormalities and Molecular Pathophysiology
 Clinical Course, Prognostic Factors, and Staging
 Treatment
 Differential Diagnosis
 CLL vs. ALL
 B-Prolymphocytic Leukemia
 Small Lymphocytic Lymphoma
 Mantle Cell Lymphoma

Small Cleaved-Cell Lymphoma
 Hairy Cell Leukemia
 Sézary Syndrome
 Adult T-Cell Leukemia/Lymphoma
 Chronic T-Cell Large Granular Lymphocytic Leukemia
 Reactive (Atypical) Lymphocytosis
 Plasma Cell Dyscrasias
Summary Chart
Case Study 21-1
Case Study 21-2
Review Questions
References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 21-1** Name the most common chronic lymphoproliferative disorder.
- 21-2** List the cellular features present in chronic lymphocytic leukemia (CLL).
- 21-3** Explain the mechanisms that cause altered immunity in patients with CLL.
- 21-4** Identify the antigens associated with B cells of interest in CLL diagnosis.
- 21-5** Recognize leukemia subtypes associated with specific patterns of cell surface marker expression.
- 21-6** Describe the detection of a clonal cell population using flow cytometric or molecular data.
- 21-7** Discuss the role of cytogenetic and molecular genetic abnormalities in the diagnosis, treatment, and prognosis of CLL.
- 21-8** Describe the clinical findings in patients with CLL.
- 21-9** List the laboratory diagnostic criteria for CLL.
- 21-10** Name the most common chromosomal abnormalities seen in CLL.
- 21-11** Describe the different disease courses of CLL.
- 21-12** Describe the treatment of CLL and the factors that drive treatment decisions.
- 21-13** Distinguish between lymphoproliferative disorders given laboratory findings.
- 21-14** Discuss the impact of laboratory tests on therapeutic decisions for the treatment of CLL.

Chronic lymphoproliferative disorders represent a heterogeneous group of diseases characterized by uncontrolled production of lymphocytes that are morphologically and immunophenotypically mature B or T or NK lymphocytes.¹ Although the features of chronic lymphoproliferative disorders are critical to the subclassification of these disorders, the final diagnosis represents a combination of morphological, immunological, cytogenetic, molecular, and clinical features. Disorders affecting bone marrow and peripheral blood are regarded as **leukemias**, whereas

diseases affecting lymph nodes and other extramedullary sites are **lymphomas**.

In recent years, considerable progress has been made in our ability to diagnose and classify hematopoietic malignancies accurately. Through cytogenetics, genomics, and molecular biology, it has been shown that many hematopoietic neoplasms are associated with a unique genotypic profile. These genetic lesions often have a direct bearing on the pathogenesis of the disease and its clinical behavior. Similarly, the development of widely available monoclonal antibodies are

enhanced molecular testing has allowed the identification of unique immunophenotypic and genetic profiles for most leukemias and lymphomas. The use of these techniques enhances both diagnostic accuracy and reproducibility.

Overview of Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL) is included in the 2016 World Health Organization (WHO) classification of mature lymphoid, histiocytic, and dendritic neoplasms.² Box 21-1 lists the mature B-cell neoplasms. Chronic lymphocytic leukemia is the most common type of leukemia in adults, and the incidence increases significantly with age. The mature B-cell lymphocytic disorders include monoclonal B-cell lymphocytosis, B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), B-cell prolymphocytic leukemia, mantle cell lymphoma (MCL), and hairy cell leukemia (HCL).² These B-cell neoplasms are often found in the peripheral blood. Other B-cell leukemias and lymphomas are discussed in Chapters 17 and 22, respectively. The mature T and NK lymphocytic disorders include adult T-cell leukemia/lymphoma, T-cell prolymphocytic leukemia, T-cell large

granular lymphocytic leukemia and aggressive NK-cell leukemia, Sezary syndrome, and Mycosis fungoides.² Refer to Chapter 22 for a discussion of lymphomas.

Normal B-Cell Development

The understanding of normal B-cell development requires the identification of different B-cell precursors. These precursors arise from hematopoietic stem cells in the bone marrow in a stepwise manner.³ Using flow cytometry, the following B-cell precursors were identified: pro-B-cells, pre-B1, pre-B2, and mature B-cells as defined by B-Cell receptor (BCR) markers with further characterization by additional markers.³ Heterogeneous phenotypes were associated with more than one B-cell maturation pathway, particularly for the pre-B1 and pre-B2 stages in which VDJ recombination takes place, with asynchronous marker expression patterns.³ VDJ recombination is the process by which B cells (and T cells) randomly assemble different gene segments known as variable (V), diversity (D), and joining (J). Next-generation sequencing in sorted B-cell precursor subsets confirms that B-cell precursor differentiation does not follow a single linear pathway.³ Rather, B-cell development is a network of parallel pathways dictated by VDJ recombination-driven checkpoints and cell signaling during B-cell production.³ B1 B cells are the closest phenotypically to the neoplastic B cells of CLL.⁴

BOX 21-1 Abbreviated List of Lymphoproliferative Disorders

Mature B-Cell Neoplasms

- Chronic lymphocytic leukemia/small lymphocytic lymphoma
- Monoclonal B-cell lymphocytosis
- B-cell prolymphocytic leukemia (as recognized by ICC)⁶
- Lymphoplasmacytic lymphoma (Waldenström macroglobulinemia)
- Plasma cell myeloma
- Monoclonal gammopathy of undetermined significance (MGUS; IgM or IgA)
- Heavy chain disease
- Solitary plasmacytoma
- Diffuse large B-cell lymphoma (DLBCL)
- Mantle cell leukemia/lymphoma
- Follicular lymphoma
- Nodal marginal zone B-cell lymphoma
- Extranodal marginal zone B-cell lymphoma
- Splenic marginal zone lymphoma
- Hairy cell leukemia
- Burkitt lymphoma

T-Cell and Putative NK-Cell Neoplasms

- T-cell prolymphocytic leukemia
- T-cell large granular lymphocytic leukemia
- NK-cell leukemia
- Aggressive natural killer (NK)-cell leukemia
- Mycosis fungoides/Sezary syndrome
- Peripheral T-cell lymphoma, unspecified
- Adult T-cell lymphoma/leukemia

Source: Swerdlow SH, Campo E, Pileri SA, Harris NL, Stein H, Siebert R, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127(20):2375-90

ADVANCED CONTENT

Classification of Lymphoid Neoplasms

There are currently two classifications of lymphoid neoplasms, one published by the World Health Organization (WHO 5th edition of the classification of lymphoid tumors)⁵ and one published by the International Consensus Classification (ICC).⁶ Both recognize CLL/SLL as disease entities.^{5,6} Both classifications recognize monoclonal B-cell lymphocytosis (MBL). The ICC recognizes B-cell prolymphocytic leukemia as an entity; however, the most recent classification by WHO does not, and this disorder is instead classified as splenic B-cell lymphoma/leukemia with prominent nucleoli.^{5,6} Various subtypes of MBL are defined in these classifications that rely on peripheral counts of neoplastic cells as well as phenotypic characteristics. In general, low-count MBL is a clonal B-cell expansion in which the clonal CLL/SLL phenotypic B-cell count is below $0.5 \times 10^9/L$ with no other features diagnostic of B-lymphoproliferative disorder. High-count or CLL/SLL-type MBL is considered a clonal expansion with $0.5\text{--}5.0 \times 10^9/L$, again with no other features diagnostic of CLL/SLL. Non-CLL/SLL-type MBL is considered "any monoclonal non-CLL/SLL phenotype B-cell expansion with no symptoms or features diagnostic of another mature B-cell neoplasm."⁵ All subtypes of MBL are clinically described by immune impairment and have an increased risk of infections.⁵ CD5, CD19, CD20, CD23, and surface or cytoplasmic kappa and lambda light chains are regarded as essential markers 1

diagnosis of CLL.^{6,7} MBL precedes virtually all cases of CLL/SLL, though MBL does not always progress to CLL.^{2,5,8} When the B-cell expansion exceeds $5 \times 10^9/L$ and the cells have the phenotypic features of CLL/SLL, the diagnosis changes to CLL. This count is arbitrary but separates groups with respect to their likelihood of requiring treatment for the disease.⁵

Hematologic Abnormalities

CLL is a B-cell neoplasm characterized by the accumulation of small, mature-appearing lymphocytes in the bone marrow, blood, and lymphoid tissues. In contrast, small lymphocytic lymphoma involves mainly lymph nodes (see Chapter 22). Neither current classification distinguishes CLL from small lymphocytic lymphoma (SLL), except by its leukemic manifestation.⁷ CLL cells proliferate in secondary lymphoid organs (lymph nodes and spleen). Here, mutation within various signaling pathways can promote the expansion of the monoclonal B lymphocytes.^{8,9}

Morphologically, the lymphocytes are small and have a hypercondensed, almost “soccer ball”-appearing nuclear chromatin pattern. “Smudge” cells are common (Fig. 21-1) and represent ruptured cells with no intact cytoplasm and nuclear structure. In CLL, smudge cells are broken CLL B cells present on the peripheral smear.

Cells present in CLL are most often indistinguishable from normal mature lymphocytes when examined with Wright stain. Prolymphocytes might be present although typically in low numbers (<2%).¹⁰ Alternatively, CLL cells can show the following:

- Increased prolymphocytes (>10%)¹⁰
- Features of activated, proliferating lymphocytes, which are larger in size and have exaggerated nuclear irregularities, open chromatin, and moderate amounts of cytoplasm¹⁰

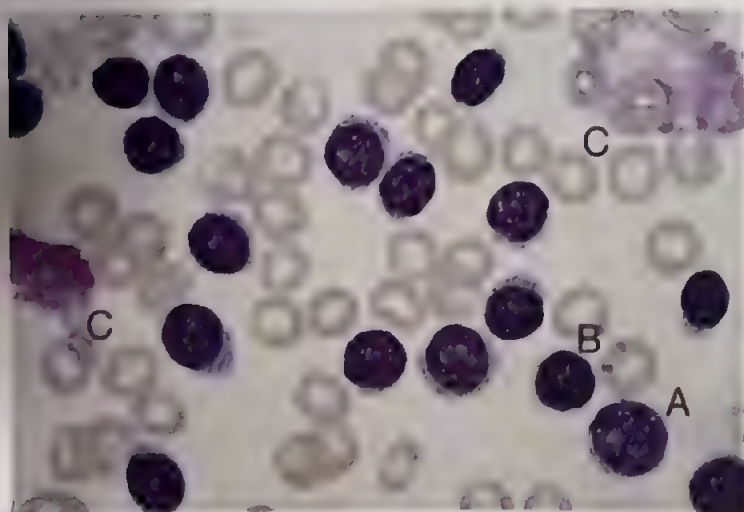


FIGURE 21-1 Photomicrograph of peripheral blood smear from a patient with chronic lymphocytic leukemia (CLL). Note the characteristic mature-appearing lymphocyte morphology with hypercondensed nuclear chromatin creating a “soccer-ball” pattern. Two smudge cells are also seen. Note the lack of platelets in this thrombocytopenic patient. A, denotes lymphoblasts; B, lymphocytes; and C, smudge cells.

- Atypical CLL, where CD19⁺ B cells are positive for CD5 but do not express (or express at very low density and in low percentage) CD23 and/or show high levels of CD20 expression (CD20 bright). The absence of t(11;14) must be confirmed to exclude mantle cell lymphoma (MCL) from the diagnosis.¹¹

Epidemiology

Patients with CLL are typically older than 50 years, and the median age of diagnosis in the United States is 72 years. Patients younger than 50 years with an absolute lymphocytosis are more likely to have a reactive lymphocytosis than CLL. Males are almost twice as likely as females to develop CLL.¹¹ CLL patients with at least one affected relative are considered to have “familial” CLL, with approximately 5% of patients reporting a history of leukemia in their family.¹ CLL is 10 to 20 times as common in Western countries than in Asia.¹¹ This suggests that other factors—genetic, environmental, or both—influence susceptibility to the disease. Often, CLL is diagnosed after a workup for incidental lymphocytosis. The presentation and clinical course are highly variable, ranging from asymptomatic, indolent disease that may never require therapy (in approximately 30% of patients) to active disease that can lead to progressive lymphocytosis, anemia, thrombocytopenia, lymphadenopathy, and hepatosplenomegaly.¹¹ Other symptoms include weight loss, night sweats, fever (B symptoms), fatigue, recurrent infections, or autoimmune complications.¹¹

Autoimmune complications, namely autoimmune thrombocytopenia and hemolytic anemia, can occur without other signs of CLL progression.^{8,12,13} Generally, 83% of patients with CLL are living 5 years after diagnosis, and more than 70% of patients can expect a survival of 10 years or more.¹⁴ This is due, in large part, to the development of targeted therapeutics.¹⁵

Etiology

No specific etiological agent or cause of CLL is currently known. A possible viral etiology has been investigated ever since the isolation of human T-lymphotropic virus type 1 (HTLV-1), a type C retrovirus from the leukemic cells of patients with T-cell malignancies.¹⁶ T-cell leukemia occurs in 3% to 5% of all carriers and usually develops after a latency period of 3 to 5 decades.¹⁶

Pathophysiology

In its classic form, this neoplastic disorder is characterized by the gradual accumulation of small mature B cells that are long-lived and immunologically dysfunctional lymphocytes in the peripheral blood and bone marrow.¹ Although the pathogenesis of the disease remains largely unknown, previous investigations have focused on defective apoptosis of the malignant cells that plays an important role in disease progression and chemotherapy resistance.¹⁷ There is additional evidence that the deregulation of cell cycle regulatory genes may contribute to the expansion of the malignant clone. CLL consists not only of resting lymphocytes but also of proliferating

leukemia cells, which have been described in proliferation centers in the lymph node and the bone marrow, and the pattern of bone marrow infiltration has a bearing on prognosis. This proliferative compartment is thought to be important for disease progression.^{2,18,19}

Additional infiltration of the lymph nodes and spleen by the malignant lymphocytes occurs in 50% of patients, whereas cutaneous invasion occurs in 25% of patients.²⁰ As the bone marrow becomes more extensively infiltrated by the leukemic clone, marrow replacement results in anemia, thrombocytopenia, and neutropenia (Fig. 21-2). Organ infiltration can lead to massive adenopathy with splenomegaly, hypersplenism, and subsequent peripheral cytopenias.²¹ An increased tendency for hemorrhage further contributes to anemia and compromises hemostasis.

Patients with CLL have significantly impaired immunological activity. The consequences of the accumulating lymphocyte mass in CLL include neutropenia, anemia, and thrombocytopenia. These may be a result of bone marrow replacement by the malignant clone or other physiological mechanisms. Lymphadenopathy, splenomegaly, or both may be present. Disruption of the immune system, beyond cytopenias, is common in CLL.^{12,13} Altered humoral immunity in patients with CLL results from suppression of all classes of immunoglobulin (Ig), leading to hypogammaglobulinemia and a subsequent increase in susceptibility to infections.^{12,13}

Another important complication of altered immunity that can develop in the patient with CLL is autoimmune disease.^{12,13} Immune dysfunction within the proliferating B cells is indicated by the presence of hypogammaglobulinemia, or hypergammaglobulinemia and monoclonal gammopathy. The frequent expression of autoantibodies in CLL contributes to a variety of autoimmune phenomena, including those leading to anemia and thrombocytopenia. The leukemic clonal B cells from CLL patients can be the source of pathogenic autoantibodies that may react with antigens present on red blood cells, platelets, or both. However, it is more commonly the nonmalignant polyclonal bystander B cells that have been shown to produce autoantibodies that contribute even more significantly to autoimmune disease.¹³

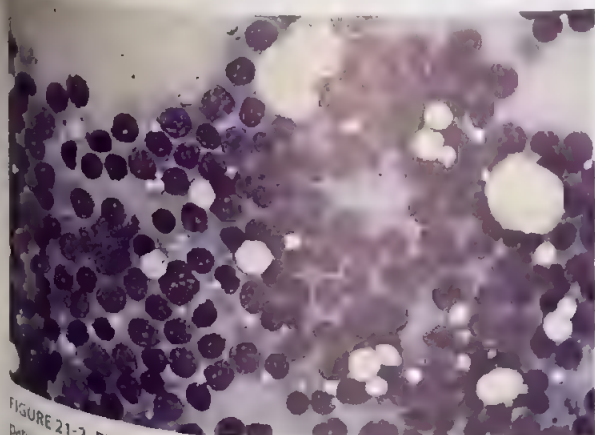


FIGURE 21-2 Photomicrograph of bone marrow aspirate smear from a patient with CLL. Note monotonous appearance of mature-appearing lymphocytes with condensed nuclear chromatin.

Hypogammaglobulinemia is found in approximately 50% of patients with CLL. The deficiency in Ig leads to infections with a variety of agents. Bacterial infections, especially of the respiratory tract, urinary tract, and skin, as well as viral infections such as herpes zoster and herpes simplex are common and dramatically contribute to patient morbidity and mortality.²²

It was found that 15% to 35% of patients develop autoimmune hemolytic anemia at some time during the course of the disease.¹³ (See Chapter 14 for more information.) Antibodies produced against red blood cells and detected with the direct antiglobulin (Coombs) test may precede, simultaneously occur with, or follow the development of CLL. Red blood cell aplasia is a rare occurrence.¹³

Autoantibodies to platelets and neutrophils may also develop and lead to immune thrombocytopenic purpura (ITP; see Chapter 26) and neutropenia. The production of autoantibodies coupled with marrow crowding and hypersplenism can lead to low peripheral platelet and neutrophil counts. Interestingly, the relatives of patients with CLL have also been shown to have an increased risk of autoimmune diseases. Immune dysfunction in some patients may include the production of paraproteins. Bence-Jones paraproteinemia has been reported in up to 49% of patients with B-CLL,¹³ and heavy-chain paraproteins, either IgM or IgG, can be detected. The pathophysiology of CLL is summarized in Figure 21-3.

CRITICAL THINKING QUESTION

21-1 If CLL is the dysregulation of B lymphocytes, and patients with CLL have increased concentrations of B cells, why is the immune response negatively impacted?

See answers to all Critical Thinking Questions at the back of this book.

Phenotypic Features and Methods for Studying Lymphocytes

In normal adult peripheral blood, an average of 25% of the circulating lymphocytes have surface Ig (sIg) and are B cells, 60% to 90% are T cells, and natural killer (NK) cells average 13%.²³ On the basis of morphological features alone, it is not possible to distinguish B cells from T cells. When a lymphoproliferative process exists, it is important to be able to characterize the nature of the lymphocytes involved. Several methods are available to study lymphocytes in lymphoproliferative disorders such as CLL (Table 21-1).

CLL is diagnosed, in a large part, morphologically. An immunophenotypic characterization of the neoplasm as either B cell or T cell using the monoclonal antibodies (mAb) available for detecting differentiation antigens (cluster differentiation CD antigens) confirms the diagnosis. The characteristic immunophenotype for B-CLL is expression of faint or low-density sIg with kappa (κ) or lambda (λ) light-chain restriction and expression of B-cell-associated antigens (CD19, CD20, CD79a), CD22, CD23, CD43, and faint CD11c (Table 21-2). An example of a B-CLL immunophenotype is shown in Figure 21-4.

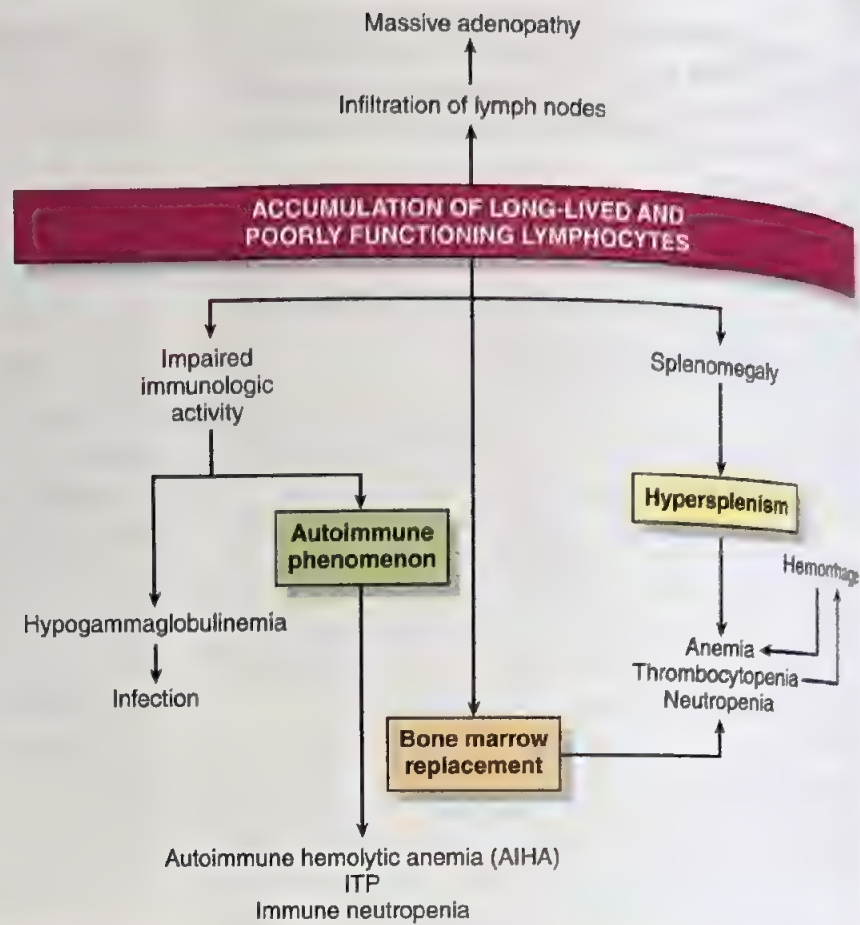


FIGURE 21-3 The pathophysiology of CLL. The three major processes that typically interact are marrow replacement by long-lived lymphocytes, hypersplenism, and autoimmunity. ITP = immune thrombocytopenic purpura.

TABLE 21-1 Methods Used to Study Lymphocytes in Lymphoproliferative Disorders

Method	Marker Detected or Feature Demonstrated
Immunophenotyping and flow cytometry	Various differentiation antigens (CD markers) on B cells and/or T cells using monoclonal antibodies
Digital Drop PCR	Rearrangements of the B-cell Ig and T-cell receptor genes, detection of minimal residual disease
Next generation sequencing	
Other molecular methods	
Cytogenetics; Fluorescent <i>in situ</i> hybridization	Consistent chromosomal abnormalities such as t(8;14)–B-ALL, Burkitt's lymphoma; t(14;18)–follicular lymphoma; 13q14 deletion–CLL, SLL; t(11;14)–mantle cell lymphoma; t(4;11)–B-ALL
Cytochemistry	Tartrate-resistant isoenzyme 5 of acid phosphatase in hairy cells Terminal deoxynucleotidyl transferase (Tdt) in B/T-ALL

T-ALL = T-lineage acute lymphoblastic leukemia; B-ALL = B-lineage acute lymphoblastic leukemia; SLL = small lymphocytic leukemia

TABLE 21-2 Immunophenotypic Features and Genetic Abnormalities of B-Lymphoproliferative Disorders

Disorder	Common Phenotype	Variable Phenotype	Associated Genetic Abnormalities
Chronic lymphocytic leukemia/small lymphocytic lymphoma	CD19+, CD20+, CD5+ , CD22(–/+), CD23+ , CD10(–), CD11c–/+, CD43+, clonal sIgM and sIgD weak; <i>BCL2</i> overexpressed	CD20+ dim Bright sIg, CD20, FMC7	Abnormalities of 13q14, 11q23, Trisomy 12, 17p-, <i>P53</i> mutation
<i>de novo</i> Prolymphocytic leukemia	CD19+, CD20+, CD5(–/+), CD22+, CD23(–), CD10(–), bright clonal sIg ; Negative Cyclin D1	CD20 and FMC7 bright	Similar to CLL, but especially <i>TP53</i> mutation

TABLE 21-2 Immunophenotypic Features and Genetic Abnormalities of B-Lymphoproliferative Disorders—cont'd

Disorder	Common Phenotype	Variable Phenotype	Associated Genetic Abnormalities
Mantle cell lymphoma	CD19+, CD20+, CD5+ , CD22+, CD23(-) CD10(-), CD43+, moderate clonal slg (IgM > IgD) BCL2 Positive, Cyclin D1 overexpressed		t(11;14)
Follicular lymphoma	CD19+, CD20+, CD5(-) , CD22+, CD10+ , CD11c(-), CD43(-), CD23(-/+), bright clonal slg	CD10 negative <20%, <i>BCL2</i> overexpressed Clonal evolution	t(14;18), del(17p)
Marginal zone and associated lymphomas	CD19+, CD20+, CD5(-/+), CD22+ CD23(-), CD10(-), CD11c+, CD25(-), CD103(-), moderate clonal slg	TRAP weak positive	Trisomy 3
Hairy cell leukemia	CD19+, CD20+, CD5(-) , CD22+ , CD23(-), CD10(-) , CD11c+, CD25+ , CD103+ , moderate clonal slg; TRAP positive		<i>BRAFV600E</i> mutation <i>MAP2K2</i> mutation
Plasma cell dyscrasias	DR(-), CD19(-), CD20(-/+), CD22(-), CD38+, CD56+/- CD138+, clonal clg	Bright CD38, CD138 and dim CD45—sensitive marker	<i>MYD88</i> mutation, add(14) (q32), total loss chromosome 13, 3q27, 17q24–25, and 20q11

Bold type indicates distinguishing feature; +/- = variable, more often positive; -/+ = variable, more often negative; (-) = negative; DR = HLA-DR; slg = surface Ig; clg = cytoplasmic Ig; *BCL2* gene expression, *TRAP* = tartrate resistant acid phosphatase.

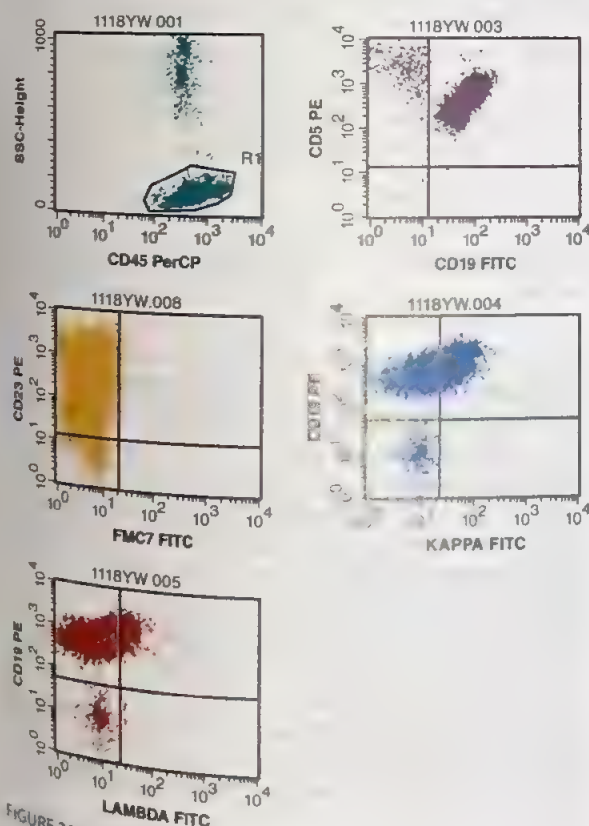


FIGURE 21-4 Flow cytometric analysis of chronic lymphocytic leukemia. Leukemia bone marrow lymphocytes are gated by CD45 scattered analysis. The plot of CD5 versus CD19 is used to demonstrate dual-positive neoplastic lymphocytes with weak CD19 fluorescence intensity. The plot of CD23 versus FMC7 illustrates positive staining of the cells for CD23 but no staining of FMC7. The cells are then plotted for both CD19 versus κ and for the dual CD19+, CD5+ cells.

In atypical CLL, CD19⁺ B cells are positive for CD5 but do not express (or express at very low density and in low percentage) CD23 and/or show high levels of CD20 expression (CD20 bright). The absence of t(11;14) must be confirmed to exclude mantle cell lymphoma (MCL) as the diagnosis.¹¹

Of particular interest is the unique expression of CD5 in B-CLL. Expression of CD5 is normally seen on T lymphocytes and on the majority of B cells of early ontogeny such as cord blood cells; however, normally less than 20% of B cells in adult human peripheral blood express CD5. The precise functional role of the CD5 molecule is unknown. CD5 has been shown to physically associate with the antigen-specific receptor complex present on both T and B lymphocytes.²⁵ As mentioned earlier, T-CLL/T prolymphocytic leukemia is rare but can be distinguished from B-CLL on the basis of differentiation antigens. Immunophenotypic marker expression and gene rearrangement during normal T-cell ontogeny helps discern the two types.

The demonstration of slg in B-CLL was mentioned earlier and is, in fact, the classic marker for B cells. The detection of a predominance of either κ or λ light chains by the B cells indicates monoclonality.²⁴ Markers for the diagnosis of CLL: CD5, CD19, CD20 and CD23 are regarded as essential.⁵ A typical example of a T-PLL immunophenotype is shown in Figure 21-5.

ADVANCED CONTENT

When the conventional immunophenotypic techniques to reveal the nature of the lymphoid neoplasm, clon

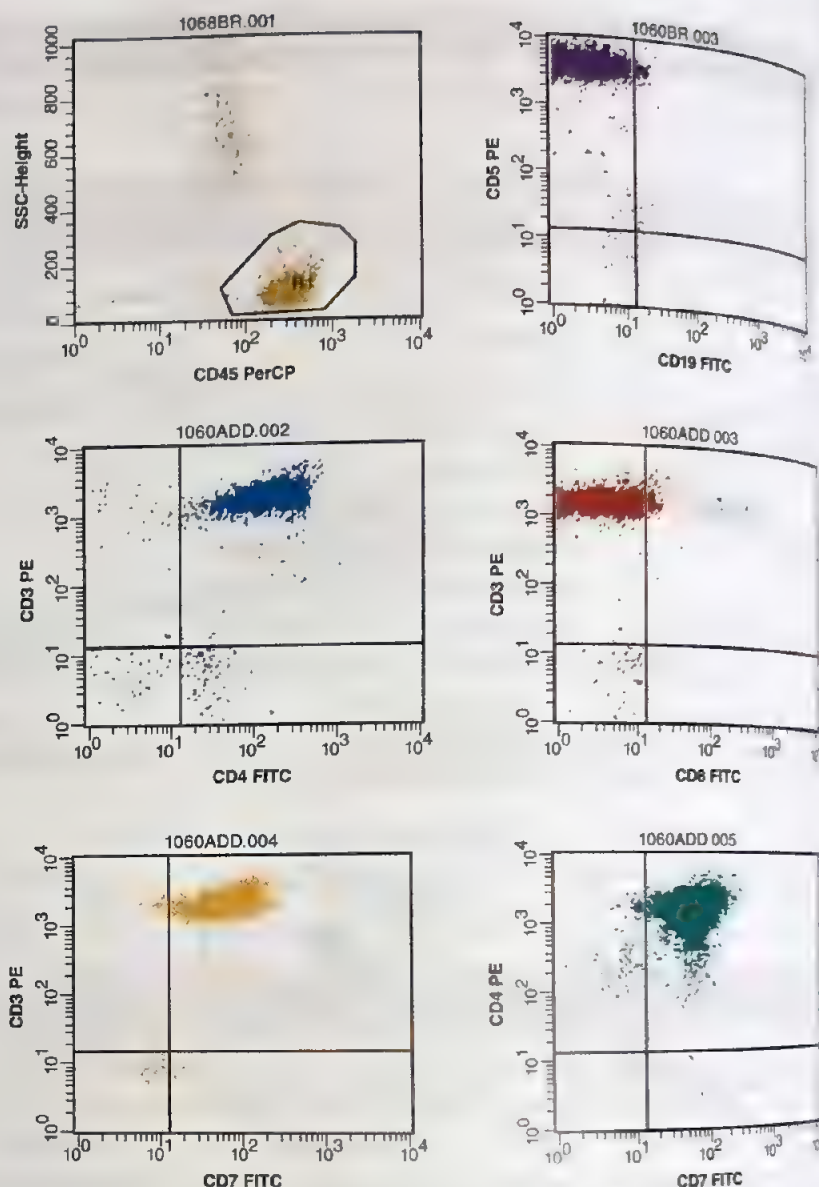


FIGURE 21-5 Flow cytometric analysis of T-prolymphocytic leukemia (T-PLL). Leukemic peripheral blood lymphocytes are gated by CD45-side scattered analysis. Comparison of CD5 versus CD19 shows a predominance of CD5-positive cells (*upper panel*). Comparisons of CD3 versus CD4 and CD3 versus CD8 demonstrate a predominance of CD4+ T lymphocytes (*middle panel*). CD4+ T lymphocytes also express CD7 antigen (*lower panel*).

a malignancy can be investigated using molecular methods. Monoclonality rather than polyclonality (the presence of a mixture of κ - and λ -bearing B lymphocytes) is a feature of many malignancies, including CLL; however, it is not, per se, indicative of malignancy. Analogous to the detection of Ig gene rearrangements in B cells is the ability to detect rearrangement patterns of the genes coding for the T-cell receptor (TCR), the antigen-specific surface molecule characteristic of T cells.^{26,29} Next-generation sequencing (NGS) has become the predominant method of evaluating the mutational status of B-cell and T-cell receptors, though recently detection of T-cell clonality has been demonstrated by flow cytometry.²⁷⁻²⁹ With NGS, the unusual case of CLL that cannot be diagnosed and classified by morphology and cell markers can now be characterized. It must be stressed that it is extremely important that the final diagnosis of any lymphoproliferative disorder be made as a result of composite information from clinical data in addition to morphological, histologic, and immunological analysis.

Although CLL is generally characterized by an elevated white blood cell count in which there are abundant lymphocytes

to analyze, if the number of neoplastic cells is low, molecular methods can be applied to improve sensitivity. Digital drop PCR combined with NGS offers the possibility of detecting low numbers of malignant clones within a complex mixture of normal cells.²⁷⁻²⁹ More commonly, real-time quantitative PCR or NGS is used.^{27,29,30} This is valuable for detecting minimal residual disease in patients who have previously been treated for CLL (or other leukemias or lymphomas) but who currently lack histopathologic evidence of relapse.^{29,30} The purpose of detecting minimal residual disease is to identify as early as possible those patients who will subsequently have a relapse but who currently have only 1% to 5% malignant cells present or have gene rearrangements present in malignant clones that require amplification to be detected.

Clinical Findings

Unlike acute leukemia, the signs and symptoms of CLL develop gradually, and the onset of the disease is difficult to pinpoint. In fact, it is not unusual for the disease to be

accidentally discovered during the course of a routine visit to a physician. The duration of a relatively asymptomatic phase of CLL is extremely variable. Unexplained absolute lymphocytosis; cervical, supraclavicular, or axillary lymphadenopathy; and splenomegaly are the earliest signs of CLL. The clinical course is indolent, but as the disease progresses, chronic fatigue, recurrent or persistent infections, and easy bruising are the consequences of anemia, neutropenia, B-cell immunological dysfunction, and thrombocytopenia. Hepatomegaly may accompany splenomegaly.^{12,13} Dermatological manifestations such as nodular and diffuse skin infiltrations, erythroderma, exfoliative dermatitis, and secondary skin infections may occur.^{19,31} Leukemic lymphocytes may invade unusual locations such as the scalp, orbits, subconjunctivae, gums, pharynx, pleura and lung parenchyma, gastrointestinal tract, prostate, and gonads.^{19,31}

Laboratory Testing and Results

The requirements for the diagnosis of CLL have undergone revision since earlier criteria were established by Rai and colleagues³² in 1975 and Binet and colleagues in 1981.³³ The International Workshop on Chronic Lymphocytic Leukemia, the World Health Organization, and the International Consensus Classification all recommend a minimum peripheral blood B-cell lymphocytosis of 5,000 cells/ μ L (5×10^9 cells/L) along with a 30% lymphocytosis of the bone marrow, consisting of morphologically mature-appearing lymphocytes.^{5,7,34}

Anemia, when it occurs, is usually normochromic and normocytic, with a normal or low reticulocyte count. Autoimmune hemolytic anemia may precede, accompany, or follow the development of CLL and be characterized by a secondary reticulocytosis, a positive direct antiglobulin test, and an elevated indirect serum bilirubin level. A decreased platelet count is not uncommon in CLL and is related to bone marrow replacement by leukemic cells, hypersplenism, and platelet antibodies.^{12,13}

The lymphocytes of CLL may be morphologically indistinguishable from normal mature lymphocytes when examined with Wright's stain. Alternatively, the leukemic lymphocytes may have exaggerated nuclear chromatin clumping with numerous dark-staining chromatin aggregates separated by light-staining areas of parachromatin. The staining pattern that results from the contrast between the nuclear chromatin and parachromatin resembles the surface pattern of a soccer ball. The morphology of peripheral blood lymphocytes in CLL is duplicated in the bone marrow aspiration and biopsy specimens. Prolymphocytes may be present and appear as medium-sized cells with a round nucleus and moderately condensed nuclear chromatin. A prominent central nucleolus should be present. There is a small to moderate amount of faintly basophilic cytoplasm.¹⁰ The percentage of prolymphocytes present correlates inversely with time to disease progression, with increased prolymphocytes suggesting more aggressive disease and a shorter time to progression.¹⁰

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The extent of marrow infiltration varies from patchy accumulations of lymphocytes to diffuse sheets that involve the entire marrow space. The pattern of bone marrow involvement is categorized into three types: nodular, interstitial, and diffuse, but combinations of these types also occur.^{11,15} The nodular pattern (Fig. 21-6) is characterized by distinct, randomly distributed aggregates of small lymphocytes. In the interstitial pattern, the lymphocytes infiltrate the interstitium to a greater or lesser degree without displacement of fat cells. The nodular and interstitial patterns are usually accompanied by preservation of normal hematopoiesis. In the diffuse pattern (Fig. 21-7), the entire bone marrow space between bone trabeculae is replaced by small lymphocytes. The life expectancy is significantly longer in patients with a nodular or an interstitial pattern than in those with a diffuse pattern.³⁵

The disruptions to the immune system stem from the interactions inherent in the complexity of the immune system coopted by the malignancy.^{12,13} Changes in the immune system affect both innate and adaptive immunity. Neutrophils, as a result of constant inflammatory signals, become nonresponsive to pathogens, resulting in enhanced risk of infection. By similar mechanisms, NK cells become nonfunctional or are induced to apoptosis during activation. The chronic cytokine profile liberated by the CLL environment serves to reduce levels of some complement components. Dendritic cells are induced to alter their phenotype to a relatively refractory state commonly found in tumor-associated macrophage. Again, through antigen-induced nonresponsiveness, regulatory cells, especially T_{reg} and myeloid-derived suppressor cells, expand and T-helper cells display markers of exhaustion. The combination of these features, along with others, serve to weaken immune surveillance, creating both an increased risk of infection due to immune suppression and an increased probability that self-reactive immune cells will escape negative selection or clonal deletion resulting in autoimmunity.^{12,13}



FIGURE 21-6 Photomicrograph of bone marrow biopsy section showing involvement by CLL, nodular pattern, characterized by distinct, randomly distributed aggregates of small lymphocytes.

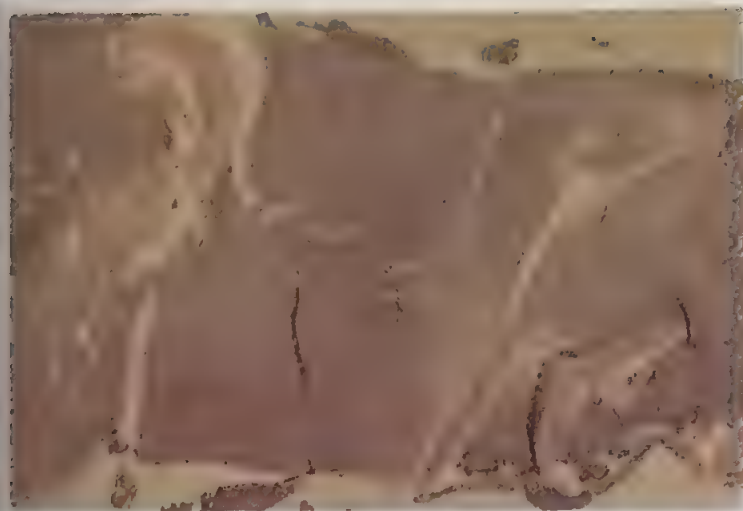


FIGURE 21-7 Photomicrograph of bone marrow biopsy section showing involvement by CLL, diffuse pattern. The entire bone marrow space between bone trabeculae is replaced by small lymphocytes.

Genetic Abnormalities and Molecular Pathophysiology

Conventional cytogenetics are inadequate for the investigation of chromosomal abnormalities in CLL as the vast majority of cells are in the G0/G1 phase of the cell cycle, are relatively quiescent, and not all changes can be visualized with G-banding. Fluorescent *In Situ* Hybridization (FISH) is used to detect both gross and cryptic chromosomal abnormalities in initial diagnosis and subsequent studies on the same patient.³⁶

The most common chromosomal abnormality in B-CLL is deletion at chromosome 13q14.^{15,37} The 13q14 region encompasses the *DLEU* locus that harbors sequences for noncoding RNAs MIR15a and MIR16-1.^{11,38} These noncoding RNAs are negative regulators of *BCL2* expression. Thus, their deletion explains, at least in part, the overexpression of *BCL2* in a majority of CLL cases. Most 13q deletions are undetectable at the cytogenetic level but are detectable in more than 50% of the CLL cases with the use of FISH for the 13q14.3 and 13q34 regions.³⁹ Patients with 13q14 deletion as the sole cytogenetic abnormality tend to have a more favorable prognosis.³⁹

Deletion in the long arm of chromosome 17 (17q-), which harbors the genomic locus of *TP53*, is found in CLL.⁴⁰ This chromosomal abnormality is identified as an independent prognostic marker for more aggressive disease. Similarly, mutations within *TP53* itself confer a less favorable prognosis.¹¹ P53 expression is stimulated by DNA damage or other oncogenic stressors, and P53 halts the cell cycle until the damage is repaired, preventing propagation of cells with chromosomal damage.⁴⁰ Loss of *TP53* or P53 function allows the cell to progress through the cell cycle in the presence of DNA damage or oncogenic stress. Loss within chromosome 11q is a recurring cytogenetic abnormality, usually associated with loss of the *Ataxia Telangiectasia Mutated* gene locus (*ATM*) and is also associated with a poor prognosis.⁴⁰ These abnormalities are related in that *ATM* is a negative regulator of MDM2. MDM2 is a ubiquitinase that ubiquitinates P53, marking it for degradation. In the absence of *ATM*, MDM2

function is no longer inhibited, and P53 is degraded even in the context of signals that would normally cause cell cycle arrest or apoptosis if P53 were present and functional.^{40,42}

Trisomy of chromosome 12 (in up to 25% of cases) is the fourth cytogenetic abnormality commonly found in CLL. This chromosomal aberration is associated with increased prolymphocytes and more rapid progression of the disease.⁴ NOTCH1/2 activation is common in conjunction with trisomy 12.⁴¹ NOTCH signaling drives proliferation of the malignant clone by directly activating cell cycle control genes as well as upregulation of cellular receptors that further drive proliferation and survival.^{37,42}

Genetic abnormalities, including the most common chromosomal abnormalities and gene rearrangements, are listed in Table 21-2. The presence of multiple chromosomal abnormalities in B-CLL has been implicated as a poor prognostic indicator. Other common genetic changes found in CLL include mutation of *SF3B1* (*Splicing Factor 3B1*), which drives utilization of cryptic splicing sites in mRNA, and mutation of *BIRC3* gene (*Baculoviral IAP repeat containing 3*), which is a negative regulator of *NFκB* (*Nuclear factor kappa B*) signaling.⁴³ None of the proto-oncogenes involved in the pathogenesis of other mature B-cell malignancies, including *BCL2*, *B-Cell Lymphoma 6* (*BCL6*), *Paired Box 5* (*PAX5*), and *c-MYC*, show frequent primary alterations in CLL.³⁷

Clinical Course, Prognostic Factors, and Staging

The overall median survival for CLL is currently estimated at 10 years; 86% of patients are living 5 years after diagnosis, whereas more than 70% have a 10-year survival.^{44,45} CLL can be an indolent disease with an asymptomatic presentation and may not require any treatment until progressive lymphocytosis of the peripheral blood and marrow, lymphadenopathy, splenomegaly, anemia, neutropenia, thrombocytopenia, autoimmune phenomena, and infection develop. This may be as late as 10 to 15 years after the initial diagnosis. In contrast to those with an indolent course of disease, approximately 20% of patients with CLL have a very aggressive clinical course that progresses rapidly from initial diagnosis and results in death within 1 to 2 years.⁴⁵ The wide variation seen among patients is not fully understood, but clinical and pathological data have been used to try to predict the CLL patient's prognosis and identify various stages and risk groups.

Treatment

Patients diagnosed with CLL in the early stages of the Rai (0-1) or Binet system (A,B) do not require immediate treatment; however, when the signs and symptoms of progressive disease appear, it is time to begin therapeutic intervention. Major physical and clinical signs and symptoms include advancing disease. These include progressive marrow failure with resulting anemia; thrombocytopenia and neutropenia; progressive lymphocytosis; progressive lymphadenopathy; enlarging spleen; autoimmunity (autoimmune hemolytic anemia or ITP); and increased susceptibility to infection and

persistent constitutional symptoms such as night sweats, fever, and weight loss.

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The Rai system,³² the Binet system,³³ and the International Workshop on CLL system³⁴ are the three major staging systems developed for CLL; however, only the Rai system is widely used in the United States. The Rai and Binet staging systems, along with median survival for each system by stage, are shown in Table 21-3. Staging systems for CLL are insufficient for predicting a clinical course as indolent or progressive. Molecular genetic factors along with clinical stage and biomarkers provide a more complete basis for prognosis. Other predictive factors for indolent CLL include blood lymphocyte doubling time (LDT) greater than 12 months⁴⁶ and a nondiffuse pattern of bone marrow lymphocyte infiltration,⁴⁷ along with a Rai stage of 0, I,

TABLE 21-3 Staging Systems for Chronic Lymphocytic Leukemia

Stage		Clinical Features
Original Rai System	Modified Rai System	
0	Low	Lymphocytosis in PB and BM ($\geq 5 \times 10^9$ lymphs/L in PB, $\geq 30\%$ lymphs in BM)
I	Intermediate	Lymphocytosis + enlarged lymph nodes
II	Intermediate	Lymphocytosis, + enlarged spleen or liver, +/- lymphadenopathy
III	High	Lymphocytosis + anemia (Hgb < 11 g/dL) +/- enlarged nodes, spleen, liver
IV	High	Lymphocytosis + thrombocytopenia (platelets < 100×10^9 /L) +/- anemia +/- organomegaly
Binet System		
A		Two or fewer node-bearing regions, no anemia or thrombocytopenia (Hgb ≥ 10 g/dL, platelets $> 100 \times 10^9$ /L)
B		Three or more node-bearing regions + no anemia or thrombocytopenia
C		Anemia and/or thrombocytopenia independent of regions involved

*Cervix, axillary, inguinal, palpable spleen and liver.
^aSurvival equivalent to that of age and sex-matched (French) population
 PB = peripheral blood; BM = bone marrow; Hgb = hemoglobin

or II. A short LDT (less than 12 months), a diffuse lymphocyte infiltration of the bone marrow (see Figure 21-7), a Rai stage of III or IV, an elevated level of serum β_2 -microglobulin, elevated level of soluble CD23 in serum, and the presence of chromosomal abnormalities are associated with a more progressive clinical course and shorter survival duration.¹⁵

Mutation of the immunoglobulin heavy chain variable region (IGHV) is an important prognostic tool.^{11,15,37,45} CLL B cells that arise before undergoing somatic hypermutation of the immunoglobulin genes (unmutated) show a different mutational landscape compared with those arising after somatic hypermutation (mutated).³⁷ "Unmutated" CLL tends to have a worse prognosis than "mutated" CLL.³⁷ High expression of ZAP-70 (a tyrosine kinase) in a subset of patients with chronic lymphocytic leukemia (CLL) is associated with an unfavorable disease outcome (Fig. 21-8).³⁸

The parameters shown in Table 21-4 have been identified as independent prognostic markers. However, the methods for measuring some of these parameters (soluble CD23, serum thymidine kinase activity) are not fully standardized and not feasible in most clinical laboratories. Further, NGS, along with other molecular methods, has demonstrated prognostic associations with changes in *Notch Receptor 1* (*NOTCH1*), *Splicing Factor 3B1* (*SF3B1*), *Baculoviral IAP repeat containing 3* (*BIRC3*), as well as *TP53*. The pace of discovery of genetic changes in this and many other diseases will continue to accelerate as more advanced studies are performed using complex molecular analyses of diseases.

Several types of transformation in B-CLL have been described, including prolymphocytoid transformation, which is relatively low grade and slowly progressive; Richter's transformation (diffuse large-cell lymphoma), which is rapidly progressive and accounts for about 5% of all deaths in CLL;^{52,53} and acute leukemia. Transformation to acute leukemia is unusual in CLL. Most patients with

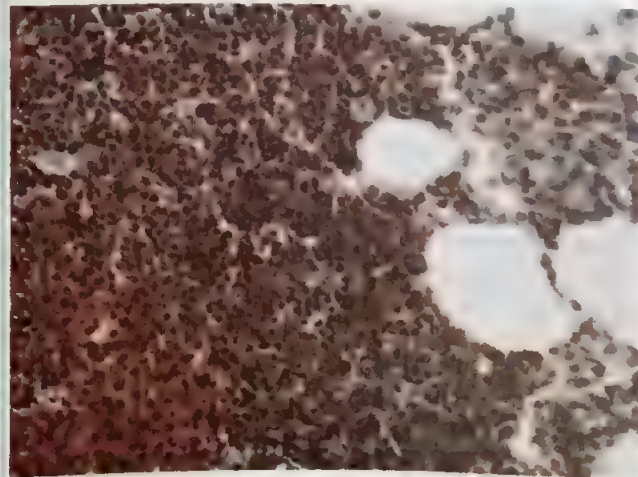


FIGURE 21-8 Immunohistochemistry ZAP-70 (Zeta-chain-associated protein kinase-70) expression in CLL cells indicates poor prognosis. Flow cytometry can also be used to detect ZAP-70 expression

TABLE 21-4 Leukemia-Cell Parameters Associated With Aggressive Disease Independent of the Disease Stage

Parameter	Reference
Deletion in chromosomes 11 (<i>ATM</i> locus) or 17 (<i>TP53</i> locus) or Trisomy 12	Kikushige, et al. ⁴ , Scarfo, et al. ¹¹ , Lee, et al. ¹⁵ , Catherwood, et al. ⁴⁰ , Hallek ³⁴ , Zakrzewska, et al. ⁴⁸ , Milne, et al. ⁴⁹ , Kittai, et al. ⁵⁰ , Swerdlow, et al. ⁵¹
Lack of somatic mutations in the expressed immunoglobulin VH-genes	Kikushige, et al. ⁴ , Scarfo, et al. ¹¹ , Lee, et al. ¹⁵ , Hallek ³⁴ , Zakrzewska, et al. ⁴⁸ , Milne, et al. ⁴⁹ , Kittai, et al. ⁵⁰ , Swerdlow, et al. ⁵¹
<i>NOTCH1</i> , <i>SF3B1</i> , <i>MYD88</i> , or <i>BIRC3</i> mutations	Kikushige, et al. ⁴ , Scarfo, et al. ¹¹ , Lee, et al. ¹⁵ , Zakrzewska, et al. ⁴⁸ , Milne, et al. ⁴⁹ , Kittai, et al. ⁵⁰
Short lymphocyte doubling time (less than 12 months)	Scarfo, et al. ¹¹ , Hallek ³⁴ , Swerdlow, et al. ⁵¹ , Morabito, et al. ⁴⁶
Elevated serum levels of β 2-microglobulin	Swerdlow, et al. ⁵¹
Elevated serum levels of soluble CD23	Swerdlow, et al. ⁵¹
Elevated serum thymidine kinase activity	Swerdlow, et al. ⁵¹
Leukemia cell-surface expression of CD38	Zakrzewska, et al. ⁴⁸ , Kittai, et al. ⁵⁰

CLL die with residual leukemia and usually succumb to infection or a cause totally unrelated to their CLL, such as cardiovascular disease.⁵³ The onset of the terminal transformation of CLL is suggested when there is a proliferation of a new population of prolymphocytes: larger cells with immature-appearing morphological features, a finer nuclear chromatin pattern, and a prominent nucleolus. This morphological transformation is often accompanied by the appearance of complex chromosomal changes that were not present earlier or are in addition to the commonly present trisomy 12. The proliferation of a more malignant clone of cells is accompanied by an increasing resistance to therapy and an exceptionally poor prognosis.^{52,53} A variety of techniques are available to help determine whether the transformation represents a clonal evolution of the original CLL or an independent disease; these include cytogenetic analysis, immunoglobulin gene rearrangement studies, and anti-idiotypic antibodies.⁵⁴

Molecular studies have shown that the development of Richter's syndrome in CLL may represent either the identical clone of cells present in the preceding CLL or a different malignant clone.⁵³ In addition, mutated *P53* (as opposed to deletion), a tumor suppressor gene that is frequently mutated in a variety of human cancers, has been discovered in 15% of CLL patients and in as high as 60% of patients with Richter's syndrome.⁵³ The close

association of *p53* with transformation of CLL into a very aggressive lymphoma is also a prognostic indicator for resistance to chemotherapy by interference with normal programmed cell death (apoptotic) pathways in tumor cells.⁵³

Treatment varies with respect to age, fitness, and molecular genetic characteristics of the disease. A host of novel targeted therapeutics have advanced the treatment of CLL and improved overall survival (Table 21-5). These include anti-CD20 antibodies, Bruton's tyrosine kinase (BTK) inhibitors, phosphoinositide 3 kinase (PI3K) inhibitors, and BCL2 inhibitors.^{49,50,52} For patients that are candidates for chemotherapy, fludarabine and cyclophosphamide are most often combined with the first generation anti-CD20 antibody rituximab (FCR) as a first-line therapy. This combination (FCR) is effective in inducing remission and 2-year progression-free survival in more than 70% of cases.⁵⁵ Alternatively, bendamustine and rituximab (BR) can be combined as a therapeutic regimen. This combination yields slightly shorter progression-free survival but is better tolerated than the FCR regimen.⁵⁶ Later generation anti-CD20 antibodies are specific for separate epitopes on the CD20 molecule and have fewer side effects compared with rituximab. These are also being investigated as first-line therapeutics.

BTK inhibitors target the essential tyrosine kinase involved in B-cell receptor signaling.⁵⁷ These inhibitors show efficacy in inducing remission in some patients alone or in combination with other drugs. PI3K inhibitors have been shown most effective in progressive disease that is unresponsive to conventional therapies.⁵⁷ BCL2 inhibitors have known toxicities and are not well tolerated in many patients. Thus, these are reserved for progressive disease in the later

TABLE 21-5 Therapeutics in Chronic Lymphocytic Leukemia

Mechanism	Therapeutic
Anti-CD20 antibodies	Rituximab Obinutuzumab Ofatumumab
Bruton's tyrosine kinase (BTK) inhibitor	Ibrutinib Acalabrutinib Zanubrutinib
Phosphoinositide-3-Kinase (PI3K) inhibitor	Idelalisib Duvelisib Umbralisib
B-cell lymphoma 2 (BCL2) inhibitor	Venetoclax
Combination chemotherapy regimens	Fludarabine/Cyclophosphamide Rituximab (FCR) Bendamustine/Rituximab (BR) Chlorambucil/Rituximab

stages of the Rai system.⁵⁷ As first-line therapy for patients with changes in the chromosome 17 or the *TP53* gene locus, DNA-damaging agents (FCR, BR) should be avoided and targeted molecular therapeutics favored to avoid exacerbating genomic instability associated with mutant *TP53*.⁵⁵ Fortunately, with the development of the targeted therapeutics, most patients can expect prolonged survival without many of the complications that plagued this disease before their development, such as massive splenomegaly requiring splenectomy or radiation therapy. The initial management of patients with CLL according to Rai staging groups is summarized in Table 21-6.

In addition to chemotherapeutic intervention, the use of intravenous gamma globulin therapy prevents major bacterial infections,¹³ and the immunosuppressant cyclosporine aids in the prevention or treatment of red cell aplasia, both of which can be management problems in patients with CLL.

Differential Diagnosis

As previously outlined in Table 21-1, CLL is a lymphoproliferative disorder that must be differentiated from other malignant or reactive lymphoid proliferations (Table 21-7). The differential diagnosis includes acute lymphoblastic leukemia (ALL); prolymphocytic leukemia (PLL) as defined by the ICC; non-Hodgkin's lymphomas in

leukemic phase, especially mantle cell lymphoma (MCL); small cleaved-cell lymphoma (SCCL); hairy cell leukemia (HCL); Sézary syndrome; T-cell large granular lymphocytic leukemia; and reactive lymphocytosis (Fig. 21-9).^{2,5,6} The morphological and immunological characteristics of these lymphoproliferative disorders are shown in Table 21-7. In addition to these disorders, other hematologic malignancies may be confused with CLL. These include adult T-cell leukemia/lymphoma (ATLL) and Waldenstrom's macroglobulinemia.⁵⁸

The diagnosis of CLL requires a sustained absolute lymphocytosis of mature-appearing lymphocytes in the absence of other causes. The diagnosis of CLL is established when the peripheral blood lymphocyte count is 5×10^9 or more cells/L (which is typically the case); lymphocyte infiltration of the bone marrow is more than 30% lymphocytes of all nucleated cells; and the circulating lymphocytes have a B-CLL immunophenotype. When lymphocyte counts are between 5×10^9 and 10×10^9 cells/L, the presence of both bone marrow infiltration of more than 30% lymphocytes and B-CLL immunophenotype (dim CD20+, CD19+/CD5+, CD23+, FMC7-, and weak surface intensity Ig) is necessary for the diagnosis of CLL.^{2,5,6}

CLL versus ALL

The distinction between CLL and ALL is easily made in most instances based on morphological differences of the proliferating cell population and immunophenotype (Fig. 21-9A and B). The difference between the smoother nuclear chromatin pattern of the lymphoblast in ALL and the heavy condensation of nuclear chromatin in the CLL lymphocyte is readily appreciated when examining an appropriate monolayer area or feather-like edge of a well-stained blood smear. Lymphoblasts show positive expression of the nuclear enzyme terminal deoxynucleotidyl transferase (TdT) and are typically CD10-positive. B-CLL cells are TdT-negative, CD10-negative, and CD5-positive.⁵¹

B-Prolymphocytic Leukemia

B-Prolymphocytic leukemia (B-PLL), as defined by the ICC, is characterized by a predominance of circulating prolymphocytes (greater than 55%, usually greater than 70%).⁶ Prolymphocytes are larger, less mature-appearing cells than the typical lymphocytes seen in CLL, with moderately condensed nuclear chromatin and a prominent vesicular nucleolus (Fig. 21-9C). The clinical and laboratory features that make PLL a distinct lymphoproliferative disorder include extreme leukocytosis (often greater than 100×10^9 cells/L) and prominent splenomegaly without lymphadenopathy.⁶ As in all disorders accompanied by leukocytosis, morphological detail of the predominating cell may not be appreciated unless appropriate areas of well-stained blood and bone marrow smears are examined. Prolymphocytes may be seen in patients with CLL but account for less than 10% of the circulating cells (Fig. 21-9D). When 11% to 55% prolymphocytes are present, a mixed-cell type of CLL, designated CLL/PLL, is diagnosed.⁶ This category includes patients with prolymphoid transformation.

TABLE 21-6 Treatment Options for Chronic Lymphocytic Leukemia

Stage	Del(17p) or Mutant TP53	IGHV Mutational Status	Treatment Options
Rai Low; Binet A-B	N/A	N/A	Observation only
Rai Intermediate-High; Binet C	Yes	N/A	Ibrutinib OR Venetoclax + Obinutuzumab OR Idelalisib + Rituximab
	No	Mutated	FCR OR Ibrutinib OR BR if >65 yrs
		Unmutated	Ibrutinib OR FCR OR BR if >65 yrs
Rai Intermediate-High; Binet C; Poor patient fitness	No	Mutated	Venetoclax + Obinutuzumab OR Chlormabmucil + Obinutuzumab OR Ibrutinib
	No	Unmutated	Venetoclax + Obinutuzumab OR Ibrutinib OR Chlormabmucil + Obinutuzumab

FCR = Fludarabine, Cyclophosphamide, Rituximab; BR = Bendamustine, Rituximab
 Credit: Adapted from Hallek, et al.⁵¹

TABLE 21-7 Morphological and Immunological Characteristics of Lymphoproliferative Disorders

Identifying Characteristic	ALL	CLL	PLL (ICC ^a)	MCL	Disorder SCCL	HCL	Sézary Syndrome	T-Gamma Lymphocytosis	Infectious Mononucleosis
Predominating or significant cell type	Lymphoblast	"Mature" lymphocyte	Prolymphocyte	"Abnormal" lymphocyte	"Abnormal" lymphocyte	Hairy cell	Sézary cell	Large granular lymphocyte	Atypical lymphocyte
Nuclear chromatin pattern	Fine	Condensed; "soccer ball"	Moderately condensed	Moderately condensed	Condensed	Fine to moderately condensed	Dark-staining	Condensed	Varies but generally less condensed than normal lymphocyte
Nuclear shape	Varies; round/oval	Regular	Regular	Irregular contours	Irregular with clefts, notches, folds	Regular to slightly irregular; may have some folding	Irregular; many folds	Regular	
Nucleoli	Prominent	Not prominent	Prominent	Not prominent	Not prominent	Not prominent	Not prominent	Not prominent	May be prominent
Cytoplasm	Scanty	Scanty	Scanty to moderate	Scant to moderate	Scanty	Moderate with hairlike projections	Scanty	Moderately abundant with prominent vacuoles and/or azurophilic granules	Abundant; may have azurophilic and/or granules vacuoles
Cell size	Varies; generally homogeneous population with some variation in size and age	Varies; generally neous population	Varies; heterogeneous population	Varies; homogeneous	Varies	Varies	Varies	Large	Large

ALL = acute lymphoblastic leukemia; CLL = chronic lymphocytic leukemia; PLL = prolymphocytic leukemia; MCL = mantle cell lymphoma; SCCL = small cleaved-cell lymphoma; HCL = hairy cell leukemia; HNK = human natural killer cell.

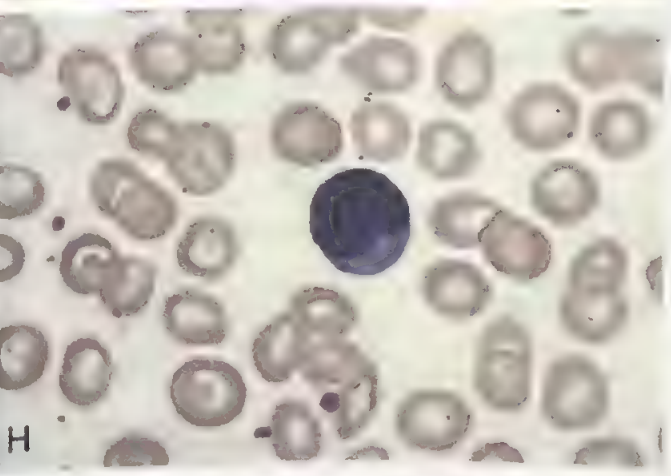
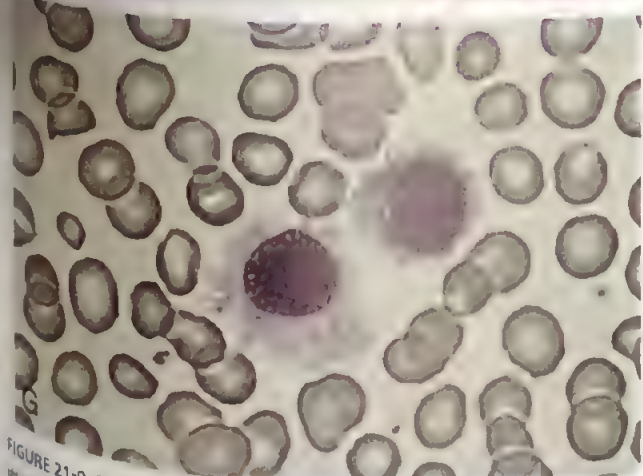
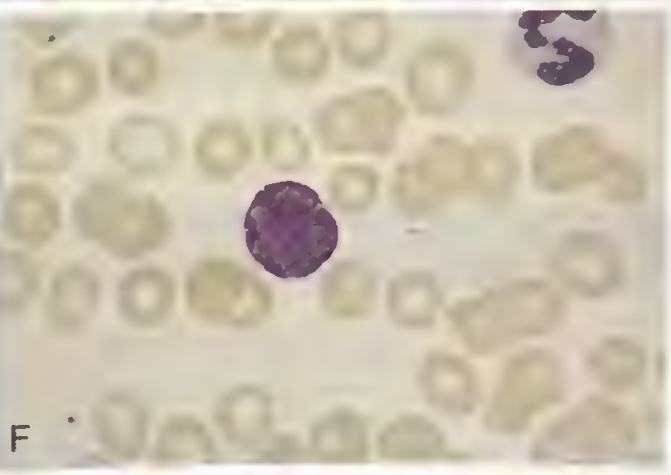
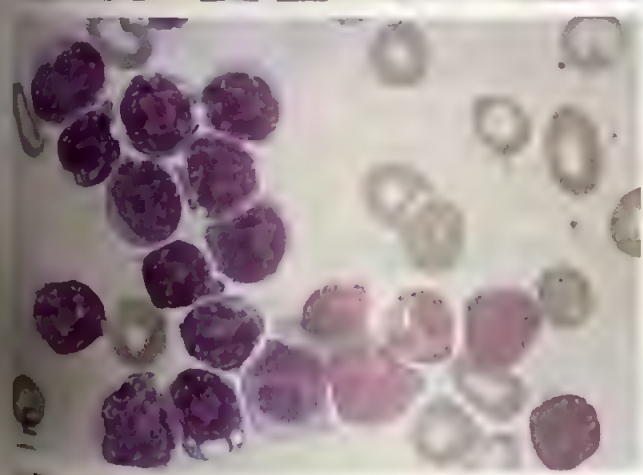
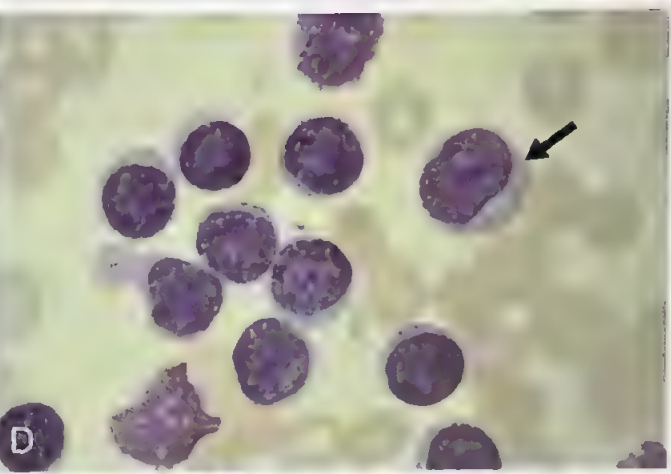
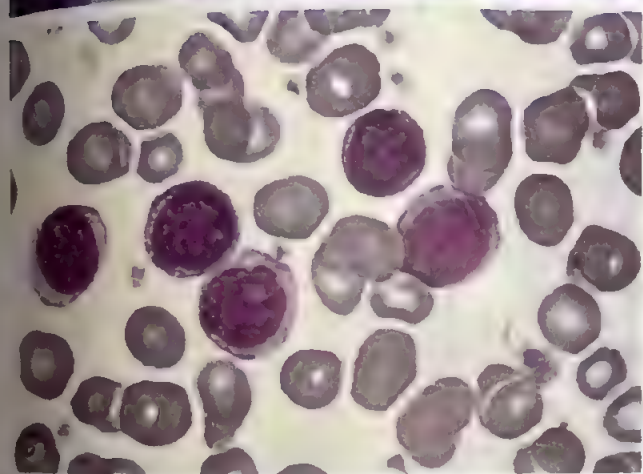
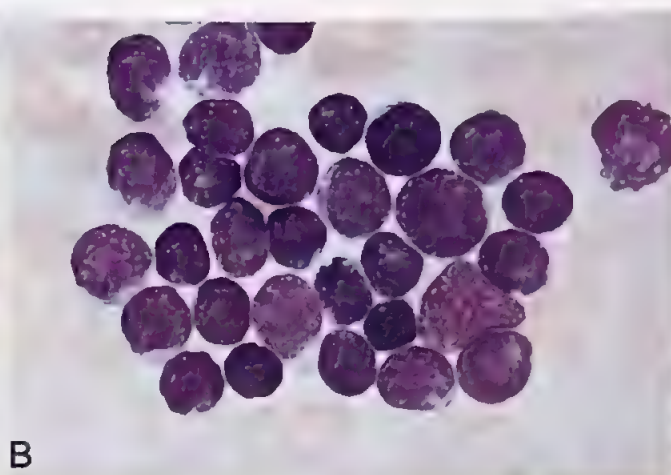
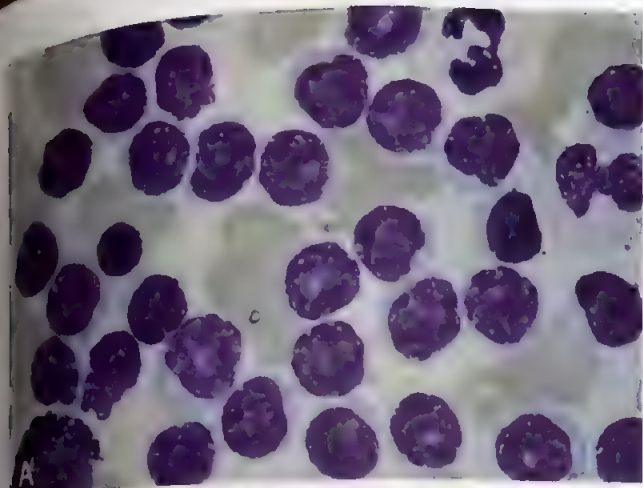


FIGURE 21-9 Peripheral blood smears of various lymphoproliferative disorders. A, Chronic lymphocytic leukemia (CLL). B, Acute lymphoblastic leukemia (ALL). C, Prolymphocytic leukemia (PLL). D, CLL with occasional polymorphocyte. E, Small lymphocytic lymphoma (SLL) in leukemic phase. F, Small, cleaved-cell lymphoma (SCCL). G, Hairy cell leukemia (HCL). H, Sézary syndrome. I, Adult T-cell leukemia/lymphoma. J, T-gamma lymphoma. K, Infectious mononucleosis with atypical lymphocytes. L, Plasma cell dyscrasia.

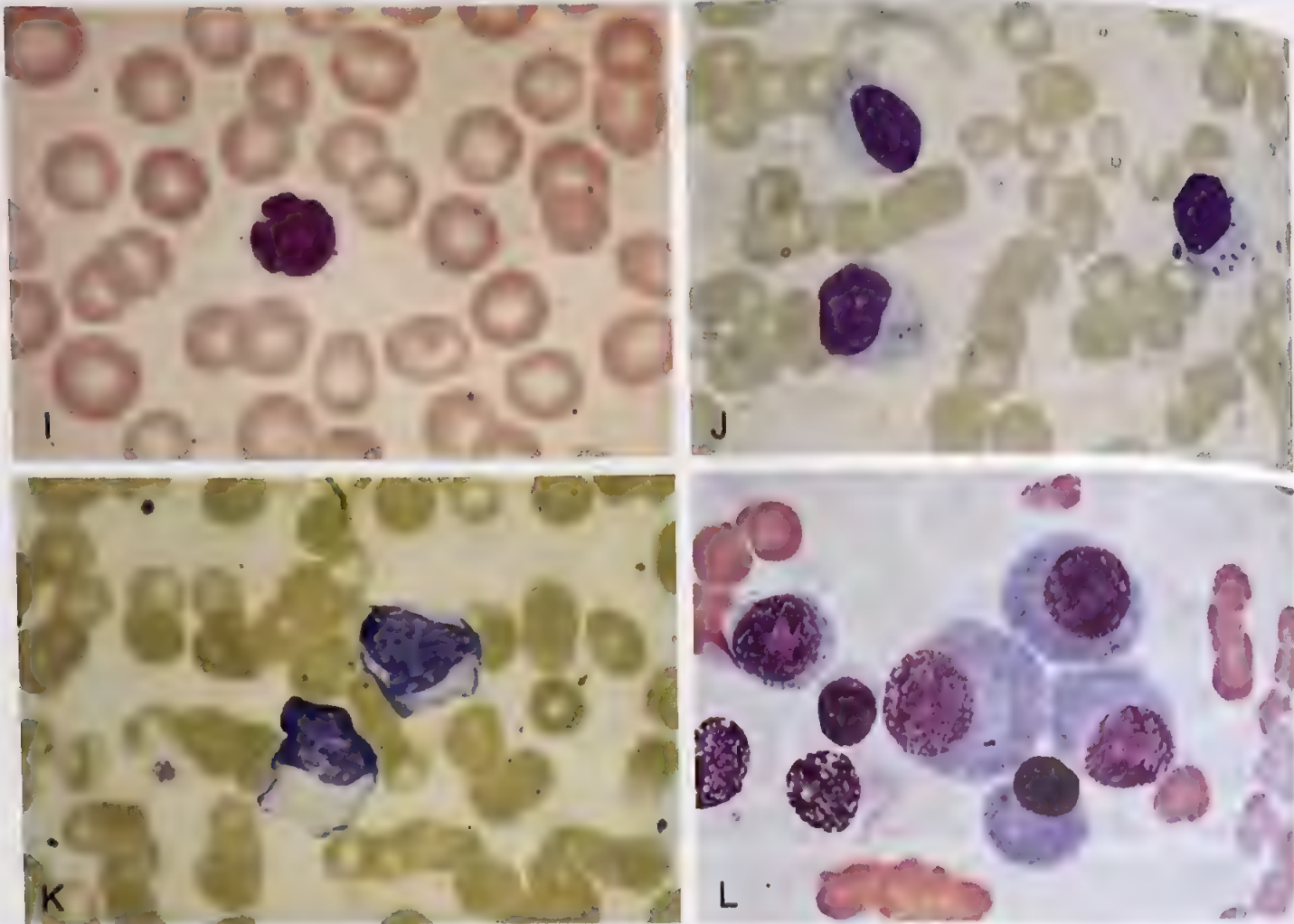


FIGURE 21-9 Cont'd

TABLE 21-8 Typical Features of Chronic Lymphocytic Leukemia

Clinical Features	Patient >50 years of Age, Lymphadenopathy, Lymphocytosis
Morphology	Mature-appearing lymphocytes in blood and marrow, often showing hyperclumped nuclear chromatin pattern; smudge cells
Immunophenotype	Positive slg, CD19, CD20, CD5, CD23, CD43
Treatment	Varies from no treatment to use of single or combination therapeutics
Prognosis	80% 5-year survival; 70% 10-year survival
Differential diagnosis	ALL, PLL, MCL, SLL, SCCL, HCL, Sézary syndrome, LGL, ATLL, Waldenstrom's macroglobulinemia, viral infection

ALL = acute lymphoblastic leukemia; PLL = prolymphocytic leukemia; MCL = mantle cell lymphoma; SLL = small lymphocytic lymphoma; SCCL = small cleaved-cell lymphoma; HCL = hairy cell leukemia; LGL = large granular lymphocytosis; ATLL = adult T-cell leukemia/lymphoma.
Source: NEW, edited Table 20-9, pg 460 from 5th ed.

ADVANCED CONTENT

Small Lymphocytic Lymphoma

Small lymphocytic lymphoma (SLL) is the nodal counterpart of B-CLL.³ It is a diffuse non-Hodgkin's lymphoma characterized by neoplastic transformation of small B lymphocytes (Fig. 21-10). Refer to Chapter 22, Lymphomas.

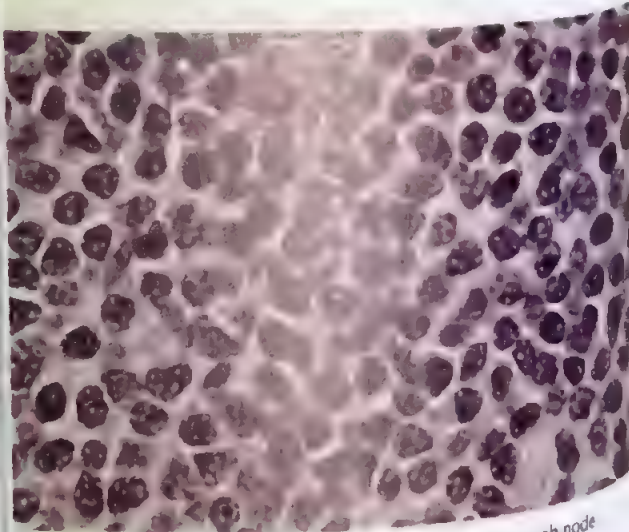


FIGURE 21-10 Small lymphocytic lymphoma (SLL), lymph node

Mantle Cell Lymphoma

Mantle cell lymphoma (MCL) arises from the centrocytes of the mantle zone of lymphoid follicles. In the lymph node, MCL usually exhibits a diffuse pattern with complete effacement of lymph node architecture.⁵⁹ It can also form a nodular or mantle zone pattern. MCL is composed of a homogeneous population of small- to intermediate-sized neoplastic lymphoid cells, usually slightly larger than normal lymphocytes. The nuclear characteristics and cytology of the cells are variable: round to clefted nuclear contour, clumped to disperse, and blastoid chromatin.⁵⁹

MCL involves the peripheral blood in up to 25% of cases, and the peripheral blood count may exceed 200×10^9 cells/L.⁵⁹ Under these circumstances, morphological recognition of MCL and its distinction from CLL/SLL

are difficult. Identification of these two entities is important because MCL is considered to have a poor prognosis, with a median survival of 3 to 5 years. Bone marrow involvement may also occur with or without peripheral lymphadenopathy. MCL shows diffuse or focal bone marrow involvement (paratrabeular and nonparatrabeular).

Neoplastic lymphoid cells in MCL cells are exclusively of B-cell lineage. The common immunophenotype of this lymphoma is shown in Table 21-4. In contrast to B-CLL, the cells in MCL lack expression of the CD23 surface antigen.⁵⁹ A typical example of the MCL immunophenotype is shown in Figure 21-11. The bright sIg expression and positivity for CD19, CD20, CD5, and FMC7 are characteristic. This immunophenotype also distinguishes MCL from follicular lymphoma, which are CD10+ and CD5+ (see Table 21-3). In most cases, a distinctive chromosomal translocation

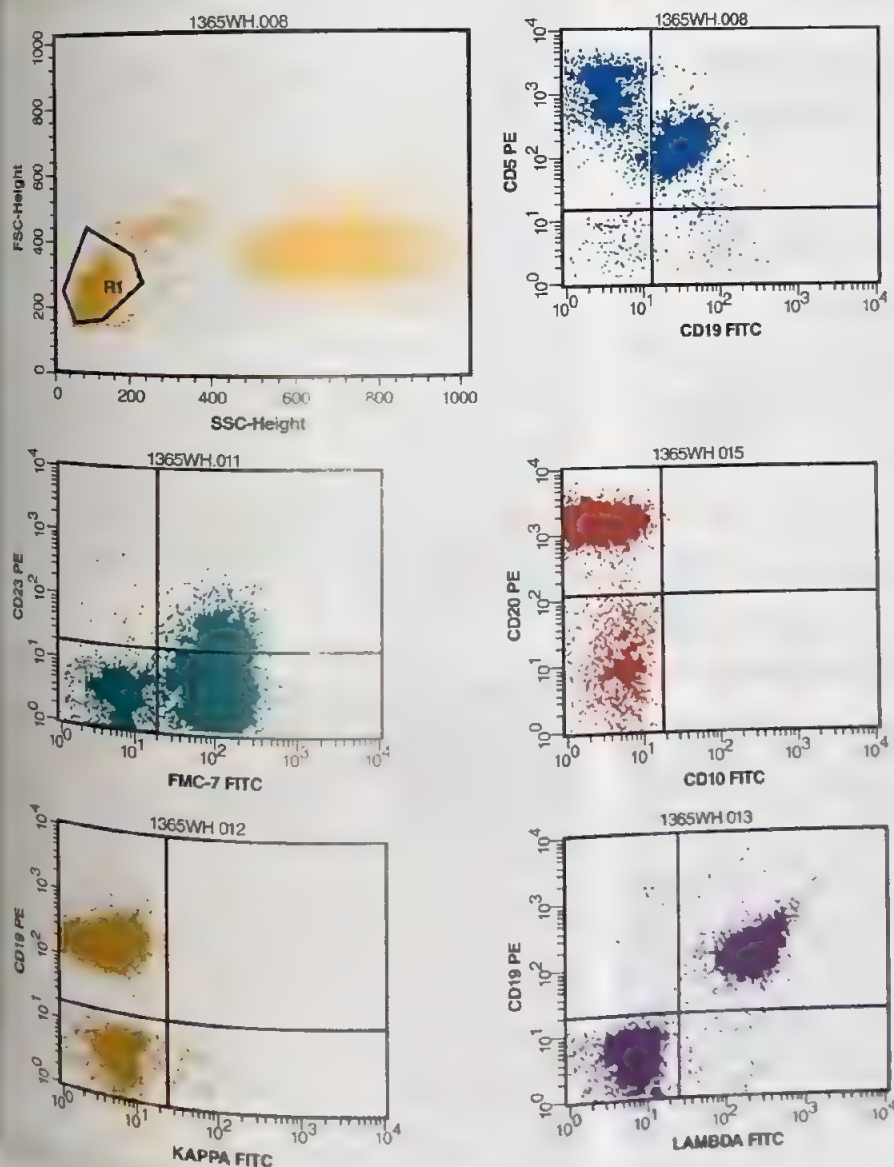


FIGURE 21-11 Flow cytometric analysis of mantle cell lymphoma in leukemia phase. Leukemic marrow lymphocytes are gated by CD45-scattered analysis. The plot of CD5 versus CD19 demonstrates dual positive neoplastic lymphocytes. In contrast to CLL, the neoplastic lymphocytes in mantle cell lymphoma show positive staining for FMC7 but no staining of CD23. Cyclin D1 immunohistochemical stain was positive.

t(11;14)(q13;q32) may occur.⁵⁸ It involves the Ig heavy-chain locus on chromosome 14 and the *BCL1* locus on the long arm of chromosome 11. The hybrid gene is associated with overexpression of the cyclin D1 (*PRAD1*) mRNA and protein. This chromosomal translocation can be detected in MCL by FISH analysis (70%) or by PCR (30% to 45%). Unlike most other B-cell neoplasms, MCL is cyclin D1- (72% to 100%) as demonstrated using immunohistochemical staining on sections of formalin-fixed tissue.^{51,59}

Small Cleaved-Cell Follicular Lymphoma

Small cleaved-cell follicular lymphoma is also a non-Hodgkin's lymphoma consisting of B lymphocytes that may be nodular (follicular) or diffuse in distribution⁵¹ (Fig. 21-12) and can progress to a leukemic phase.⁵¹ The circulating cells of SCCL are morphologically characterized by nuclei that are irregular in shape and that demonstrate irregular clefts, notches, or folds that may traverse the entire width of the nucleus (Fig. 21-9F). These abnormal lymphocytes typically have very scanty cytoplasm. Compared with immunological markers in B-CLL, SCCL shows strong slg, CD22 positivity, CD5 negativity, and often CD10 positivity.⁵¹

Hairy Cell Leukemia

Hairy cell leukemia is another form of B lymphocyte-derived chronic leukemia and so named because of the fine, hairlike, irregular cytoplasmic projections that typify the disease (Fig. 21-9G). Pancytopenia is common in HCL (unlike the other lymphoid disorders discussed in this chapter), along with splenomegaly and marrow fibrosis. Monocytopenia is almost universally found in the peripheral blood of HCL patients.⁶⁰ A bone marrow aspirate is often difficult to obtain because of associated fibrosis (the so-called dry tap). Nevertheless, bone marrow biopsy and biopsy touch imprints are essential for diagnosis. The bone marrow biopsy shows a loose interstitial lymphocytic infiltrate surrounded by a clear cytoplasm that separates one cell from another, creating a "fried-egg" appearance (Fig. 21-13). The most characteristic cytochemical feature of HCL is a strong acid phosphatase reaction that is

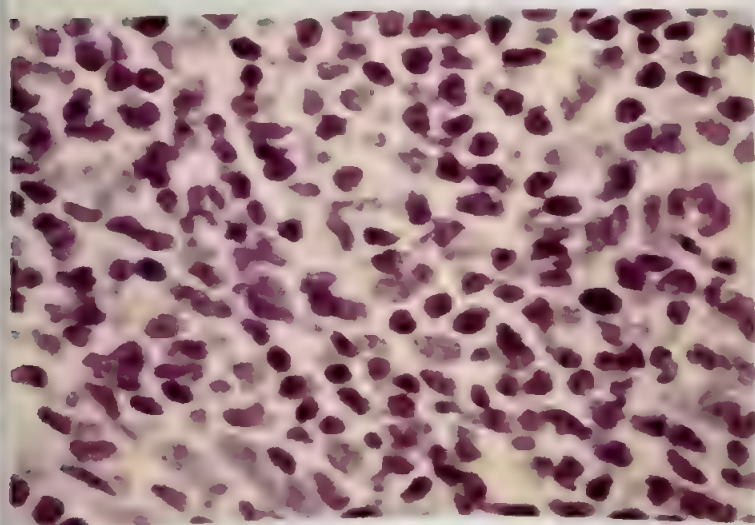


FIGURE 21-12 Small cleaved-cell lymphoma (SCCL) lymph node

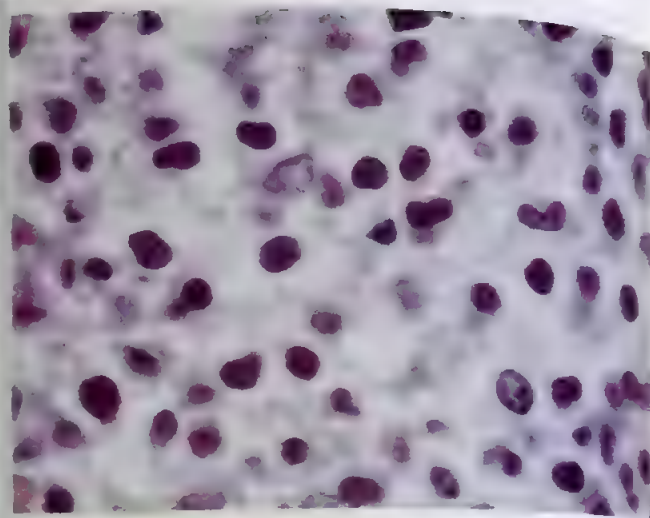


FIGURE 21-13 Hairy cell leukemia (HCL) bone marrow aspirate

not inhibited by tartaric acid, known as the *tartate-resistant acid phosphatase* (TRAP) stain (Fig. 21-14).⁵ Immunological markers that support the diagnosis of HCL are reactivity with B-cell-associated antigens (CD19, CD22, CD79a), CD11c, CD25 (the monoclonal antibody that recognizes the interleukin-2 receptor, Tac), FMC1, and CD103.⁶⁰ CD103 is the most useful marker for distinguishing HCL from other B-cell leukemias. A typical example of the HCL immunophenotype is shown in Figure 21-15. A variant of HCL with splenomegaly and leukocytosis has been described in the ICC⁶ but is not recognized by the current WHO classification.⁵

A majority of HCL cases harbor the *BRAF* V600E mutation. Many cases lacking *BRAF* mutation demonstrate mutations in the downstream kinase *MAP2K1* in variant HCL.⁶⁰ Although targeted molecular therapeutics are in development for mutant *BRAF*, conventional therapies using nucleoside analogs are currently able to induce stable remission without significant development of drug resistance.⁶⁰



FIGURE 21-14 Tartrate-resistant acid phosphatase (TRAP) stain of peripheral blood showing positivity in hairy cell and no staining in neutrophils

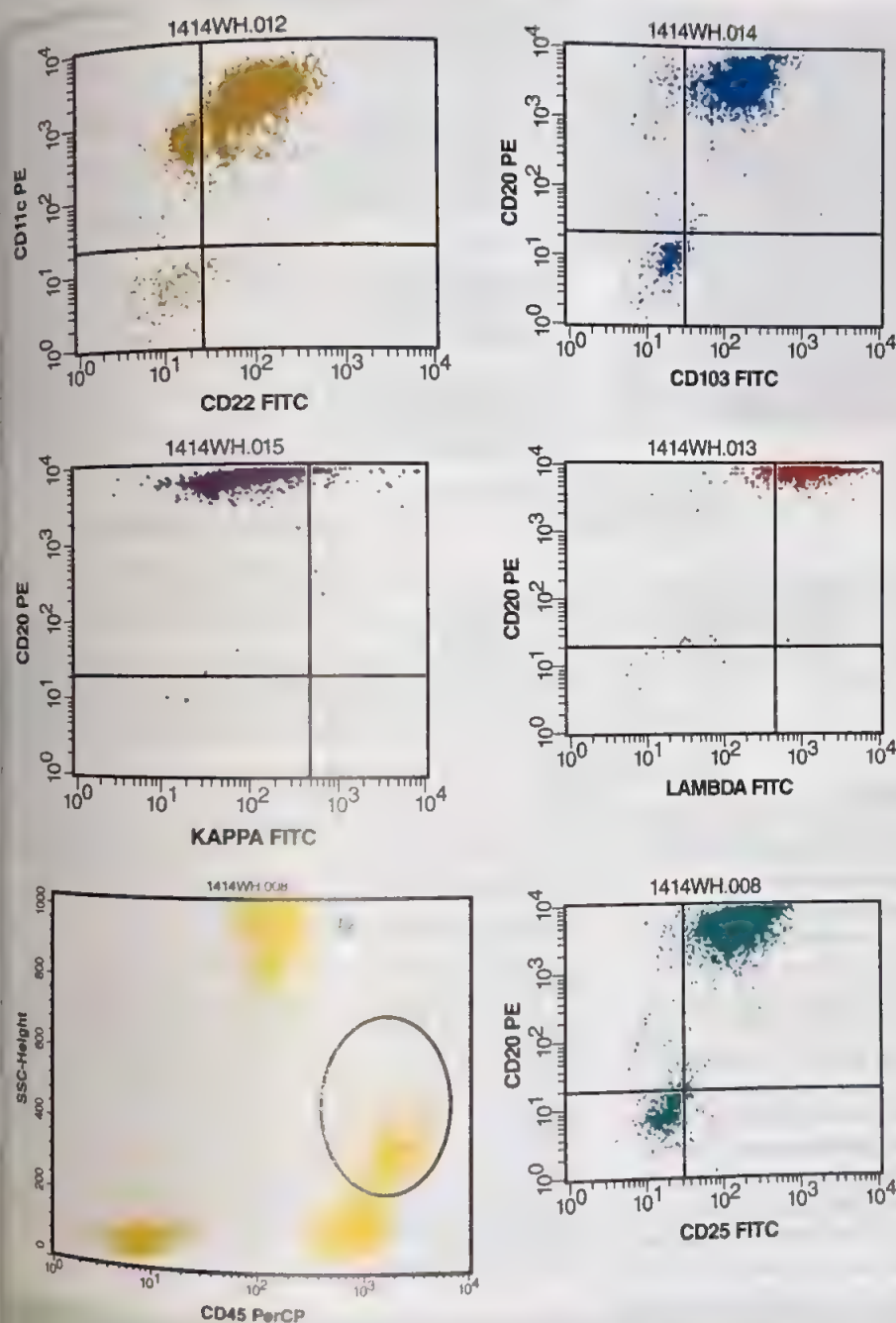


FIGURE 21-15 Flow cytometric analysis of hairy cell leukemia. Large mononuclear cells in leukemic bone marrow are gated by CD45-side scattered analysis (left lower panel). The plots of CD22 versus CD11c and CD20 versus CD103 demonstrate predominance of dual positive B cells (upper panel). The plots for both CD20 and λ show λ light-chain clonality (middle panel). The B cells are also reactive with anti-CD25 (anti-interleukin-2 receptor) (lower right-sided histogram).

Sézary syndrome

Sézary syndrome is the leukemic phase of the most common cutaneous T-cell lymphoma, mycosis fungoides, and is characterized by abnormal circulating lymphocytes, called Sézary cells.³¹ A Sézary cell is typically the size of a small lymphocyte and has a dark-staining, hyperchromatic, nuclear chromatin pattern with numerous folds and grooves referred to as cerebriform (Fig. 21-9H). Nuclear folding is best appreciated at the ultrastructural level using electron microscopy. A less common large cell variant of the Sézary cell is larger than a neutrophil and often larger than a monocyte

but has the same grooved nuclear chromatin pattern as the smaller Sézary cell. The bone marrow is infrequently involved. The diagnosis of Sézary syndrome is dependent on the primary diagnosis of the cutaneous T-cell lymphoma mycosis fungoides in the skin. The skin biopsy shows infiltration in the epidermis with an accumulation of atypical, convoluted lymphocytes forming structures called Pautrier microabscesses (Fig. 21-16). Immunological marker studies of Sézary cells show a mature T-lymphocyte phenotype with reactivity for CD2, CD3, and CD4 (the monoclonal antibody that recognizes the helper/inducer subset of

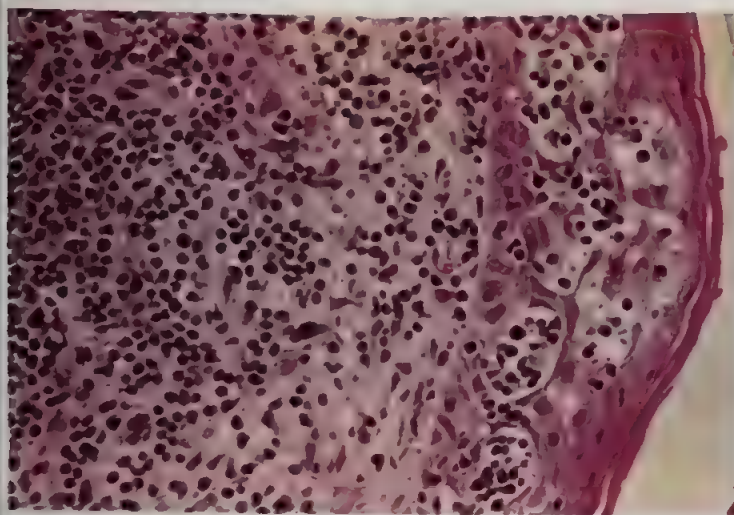


FIGURE 21-16 Infiltration of the epidermis and upper dermis by lymphocytes, many with convoluted (cerebriform) nuclei, histiocytes, and formation of Pautrier microabscesses, characteristic of the cutaneous T-cell lymphoma mycosis fungoides.

T lymphocytes). The detection and amplification of markers of T-cell clonality at the TCR and alpha-beta ($\alpha\beta$) and gamma-delta ($\gamma\delta$) loci by PCR can significantly improve the sensitivity in detecting a clonal T-lymphoid population.²⁷

Adult T-Cell Leukemia/Lymphoma

Adult T-cell leukemia/lymphoma, common in Japan and the Caribbean, is caused by human T-cell leukemia/lymphoma virus-1 (HTLV-1). Although there is a large heterogeneity in the clinical manifestations of the disease, characteristic clinical features include generalized lymphadenopathy, hypercalcemia, bone and skin lesions, and 10% to 80% abnormal lymphoid cells in the blood and bone marrow.¹⁶ The most outstanding feature of these abnormal lymphocytes is the highly convoluted nuclear shape, which often is “cloverleaf” in appearance (Fig. 21-9J). There is marked variation in the size of the cells, ranging from that of a small lymphocyte to that of a large monocyte. Nucleoli are typically inconspicuous but, when present, may be prominent and cause confusion with prolymphocytes. The clinical course of ATLL can be acute, with a high white blood cell count and survival less than 1 year; chronic, with a lower white cell count and survival of more than 1 year; or “smoldering,” with a normal white cell count and low numbers of abnormal T lymphocytes. As seen in T-PLL and Sézary syndrome, ATLL cells show reactivity with T-cell-associated antigens (CD2, CD3, and CD5); most are CD4+, CD25+ but usually lack CD7.⁶¹ Rare CD8+ cases have been reported.⁶¹ Mutation in the tumor suppressor gene *p53* is seen in 30% to 50% of patients with ATLL.⁵¹

Chronic T-Cell Large Granular Lymphocytic Leukemia

Chronic T-cell large granular lymphocytic leukemia (LGL) has the morphological distinction of persistent circulating lymphocytes that have abundant pale blue cytoplasm with azurophilic granules (Fig. 21-9J). Three distinct clinical syndromes are now described in patients with an increased

number of circulating LGL cells. When LGL carries a phenotype of T-LGL leukemia (a clonal proliferation of CD3+ LGL), then chronic neutropenia and autoimmunity, especially rheumatoid arthritis, are characteristic.⁵¹ Natural killer LGL leukemia is characterized by a clonal CD3– LGL proliferation with an aggressive clinical course and multiorgan involvement.¹ The majority of patients with increased numbers of CD3+ cells do not have features of NK-LGL leukemia but rather demonstrate a more indolent clinical course.⁵¹ Because quantitative abnormalities of LGL are fairly common and their presence in peripheral blood may represent a transient reactive phenomenon associated with viral infections, it is important to perform immunophenotyping and molecular studies, and to correlate these data with the clinical picture. Oral low-dose methotrexate alone or in combination with other agents has been shown to be an effective treatment for some patients with LGL.⁵¹

Reactive (Atypical) Lymphocytosis

Reactive (atypical) lymphocytosis is self-limiting, rarely exceeds 5×10^9 cells/L, and is most commonly caused by a viral infection such as infectious mononucleosis, viral hepatitis, and cytomegalovirus in adults and *Bordetella pertussis* in children. The large reactive lymphocytes that characterize viremia are polyclonal and T cell in origin. Abundant cytoplasm that may vary in degree of basophilia from very pale to deep blue is the most prominent feature of the reactive lymphocyte. These cells often have an irregular nuclear outline resembling a monocyte, and the nuclear chromatin is mostly coarse (Fig. 21-9K). Reactive B-cell lymphocytosis is rare. See Chapter 16, which discusses infectious mononucleosis and other causes of reactive lymphocytosis.

Plasma Cell Dyscrasias

Plasma cell dyscrasias, namely Waldenström's macroglobulinemia, multiple myeloma, and plasma cell leukemia, may be associated with the presence of abnormal circulating plasma cells (Fig. 21-9L). Plasma cells are characterized by abundant basophilic cytoplasm, an eccentric nucleus with clumped nuclear chromatin, and a prominent perinuclear clear zone. Plasma cells are end-stage B lymphocytes with the aforementioned characteristic morphology and distinctive immunological markers, namely, the presence of monoclonal cytoplasmic Ig and expression of CD38. Plasma cell disorders are covered extensively in Chapter 23.

CRITICAL THINKING QUESTION

21-2 Morphologically, how can lymphoproliferative disorders be distinguished from one another?

The significant clinical, morphological, and immunophenotypic features of CLL, as well as treatment, prognosis, and differential diagnosis, are shown in Table 21-8. A general approach to the differential diagnosis of lymphocytosis is shown in Figure 21-17.

In summary, B-cell small lymphocytic disorders are chronic diseases that usually present in an indolent fashion in older

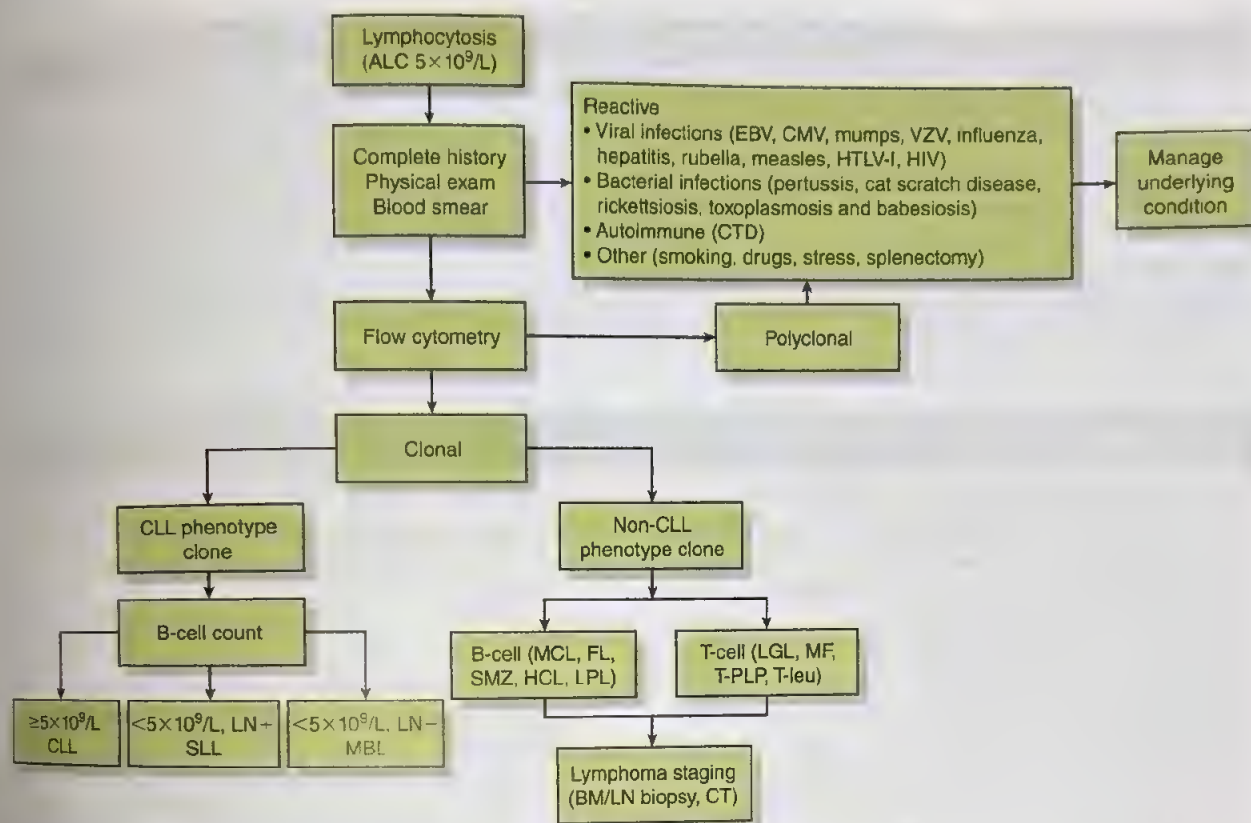


FIGURE 21-17 General approach to the workup of lymphocytosis. ALC, absolute lymphocyte count; BM, bone marrow; MBL, monoclonal B cell lymphocytosis; CMV, cytomegalovirus; CTD, connective tissue disease; EBV, Epstein-Barr virus; FL, follicular lymphoma; HCL, hairy cell leukemia; HTLV, human T-lymphotropic virus; LGL, large-granular leukemia; LN, lymph nodes; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; MF, mycosis fungoides; T-PLP, T-prolymphocytic leukemia; SMZ, splenic marginal zone lymphoma; T-leu, T-cell leukemia; VZV, varicella zoster virus. Used with permission from Paolo Strati, Tait D. Shanafelt. Monoclonal B-cell lymphocytosis and early stage chronic lymphocytic leukemia: diagnosis, natural history, and risk stratification. *Blood* 2015;126(4):454-462.

patients. The diagnosis is often made incidentally from a persistent lymphocytosis. B-cell CLL is a clonal accumulation of mature-appearing B lymphocytes caused by failed apoptosis, exhibiting monoclonal sIg. An unusual feature of CLL is expression of the CD5 membrane antigen normally seen on mature T lymphocytes. The B lymphocytes accumulate slowly in the bone marrow, blood, spleen, liver, and lymph nodes. About half of the patients with B-CLL exhibit hypogammaglobulinemia and an increased susceptibility to infection. Red

cell aplasia, immune-mediated anemia, and thrombocytopenia are also common.

The course of CLL disease depends on the leukemia burden, which can be assessed by clinical staging systems.

Lymphoproliferative disorders caused by T lymphocytes are rare. T-prolymphocytic and ATLL are rapidly progressive diseases with more aggressive clinical courses. Morphological and immunophenotypic studies, and positive HTLV-1 serology, are helpful in characterizing these entities.

SUMMARY CHART

- Chronic lymphoproliferative disorders are clonal proliferations of morphologically and immunophenotypically mature B or T lymphocytes.
- Most CLLs are disorders of B lymphocytes.
- The Rai system, the Binet system, and the International Workshop on Chronic Lymphocytic Leukemia (CLL) are the three major staging systems developed for CLL.
- The clinical course of CLL is indolent, but as the disease progresses, chronic fatigue, recurrent or persistent

infections, and easy bruising resulting from anemia, neutropenia, B-cell immunological dysfunction, and thrombocytopenia may occur.

- Laboratory findings in CLL include normocytic, normochromic red cells. Lymphocytes are small or slightly larger than normal size and have a relatively, well-differentiated appearance. Autoimmune hemolytic anemia may precede, accompany, or follow the development of CLL and is characterized by secondary

SUMMARY CHART—cont'd

reticulocytosis, a positive direct antiglobulin test, and an elevated indirect serum bilirubin level.

- B lymphocytes in CLL patients have dysfunctional immunoglobulin production, making patients more susceptible to bacterial and viral infections.
- Differential diagnosis includes acute lymphoblastic leukemia (ALL), prolymphocytic leukemia (PLL),

non-Hodgkin's lymphomas in leukemic phase, mantle cell lymphoma (MCL), small cleaved-cell lymphoma (SCCL), hairy cell leukemia (HCL), Sézary syndrome, T-cell large granular lymphocytic leukemia, and reactive lymphocytosis.

CASE STUDY 21-1

A 74-year-old black woman was admitted to the hospital for high fever. Her past medical history revealed chronic obstructive pulmonary disease, hypertension, sickle cell trait, and CLL. The CLL had been diagnosed 13 years ago and treated with chlorambucil, prednisone, vincristine, and bleomycin. She had a fairly recent bone marrow analysis that had shown no lymphoid infiltrates. On admission she was confused, minimally communicative, and weak. Lab results revealed a high white blood cell count, and slightly low red blood cell count and platelet count. Hemoglobin and hematocrit were also slightly decreased. Differential white blood cell evaluation showed increased neutrophils, including increases in bands, as well as mild lymphocytosis. A few segmented neutrophils were observed phagocytizing bacteria.

The patient was thought to be clinically septic and was started on clindamycin, gentamicin, and ampicillin therapy. Blood and urine Gram stains were positive.

QUESTIONS:

1. Are this patient's lab results consistent with her CLL diagnosis?

2. Why might the patient have a bacterial infection?
3. If the patient has increased neutrophils and lymphocytes, why isn't she able to fight the bacterial infection?

ANSWERS:

1. Yes, it appears the patient has a chronic version of CLL, which means she is living with well-managed slight anemia.
2. CLL patients are more susceptible to bacterial infection because of compromised humoral immunity.
3. The B lymphocytes in CLL patients have a dysfunction that affects the production of immunoglobulins.

Note: This case illustrates how susceptibility to opportunistic infections and immunological deficiency can affect a patient with CLL. Although patients with CLL frequently have a prolonged survival and succumb to an unrelated disorder, the course of CLL varies widely in different patients.

CASE STUDY 21-2

A 64-year-old man with no significant past medical history was admitted to the hospital with mild upper abdominal pain, malaise, anorexia, weight loss, and low-grade fever of 1-month's duration. He denied having a cough, dyspnea, nausea, vomiting, jaundice, or recent viral infection. His vital signs were essentially unremarkable except for a mild fever of 37.8°C. Physical examination revealed marked splenomegaly with no hepatomegaly or peripheral lymphadenopathy. Radiological examination, however, disclosed retroperitoneal lymphadenopathy. Laboratory workup showed marked leukocytosis (182×10^9 cells/L), thrombocytopenia (71×10^9 cells/L), and anemia (hemoglobin, 8.1 g/dL). Peripheral blood and bone marrow aspirate smears were performed. More than 60% of the leukocytes counted in the peripheral blood and 85% of all nucleated

cells counted in the bone marrow specimen were described as "medium to large atypical cells with distinct rim of cytoplasm, round nuclear contour, dispersed nuclear chromatin, and a single prominent nucleolus."

QUESTIONS:

1. Based on the level of leukocytosis, which lymphoproliferative disorder is likely present?
2. Do the differential findings align with this potential diagnosis?

ANSWERS:

1. PLL
2. Yes, the lymphocytes are larger. There is dispersed nuclear chromatin and a prominent nucleolus.

REVIEW QUESTIONS

- Chronic lymphocytic leukemia is most commonly a neoplasm of which white blood cell?
 - T lymphocytes
 - Neutrophils
 - B lymphocytes
 - Monocytes
- Which of the following statements is true of smudge cells?
 - They are larger than normal lymphocytes.
 - They are an artifact resulting from peripheral smear slide preparation.
 - They are soccer ball–appearing chromatin patterns.
 - They are actually monocytes.
- CLL cells typically present with features of activation, which include:
 - Lymphocytes smaller in size
 - Exaggerated chromatin patterns
 - Lobulated or deep nuclear folds
 - Little to no cytoplasm
- Altered immunity in CLL is caused by which of the following?
 - Neutophilia
 - Increased production of immunoglobulins
 - Common development of autoimmune disorders
 - Anemia
- Which of the following is a B-cell marker?
 - CD 19
 - CD 21
 - CD 8
 - CD 4
- How does CLL typically present?
 - Women under 50 years old
 - Acutely with aggressive symptoms
 - Clearly pinpointed beginning of disease
 - Men over 50 years old
- Which is a laboratory finding consistent with a CLL diagnosis?
 - Lymphocyte count of $2 \times 10^9/L$
 - Bone marrow lymphocyte count of 30%
 - High red blood cell count
 - Normal platelet count
- Which is a common B-CLL chromosomal abnormality?
 - 13q14 deletions
 - Trisomy 12
 - 17q translocation
 - TP53 deletion
- Prognosis is decreased in CLL patients that present:
 - With indolent disease with asymptomatic presentation
 - With minimal lymphocytosis
 - With aggressive progression of lymphocytosis, anemia, and autoimmune phenomenon
 - With no anemia
- Which treatment works best in progressive disease unresponsive to other therapies?
 - Anti-CD20
 - BCL2 inhibitors
 - BTK inhibitors
 - P13K inhibitors
- Lymphocytosis in peripheral blood and bone marrow with CD 20 +, CD 19 +, CD 5 +, and CD 23 + indicates which of the following lymphoproliferative disorders?
 - ALL
 - PLL
 - CLL
 - SLL
- Lymphoblasts are seen in which lymphoproliferative disorder?
 - ALL
 - PLL
 - CLL
 - SLL
- The cells of chronic lymphocytic leukemia are morphologically identical to those of:
 - Acute lymphoblastic leukemia
 - Small lymphocytic lymphoma
 - Infectious mononucleosis
 - Sézary syndrome
- Surface immunoglobulin is the most reliable surface marker for:
 - T lymphocytes
 - Plasma cells
 - B lymphocytes
 - Histiocytes
- Cells that demonstrate a positive reaction with the tartrate-resistant acid phosphatase (TRAP) stain are most likely:
 - T lymphoblasts of acute lymphoblastic leukemia
 - Atypical lymphocytes of a viral infection
 - Large granular lymphocytes of T-gamma lymphoproliferative disorder
 - Hairy cells of hairy cell leukemia

See answers at the back of this book

The Lymphomas

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CHAPTER OUTLINE

Hodgkin Lymphoma

Epidemiology, Etiology, and Pathogenesis

Pathology

Clinical Findings

Staging and Treatment

Non-Hodgkin Lymphoma

Epidemiology, Etiology, and Pathogenesis

Pathology

B-Cell Lymphomas

T-Cell and Natural Killer (NK)-Cell

Lymphomas

Histiocytic and Dendritic Cell Tumors

Diagnostic Evaluation of Lymphoid

Neoplasia

Clinical Findings

Treatment and Prognosis

Summary Chart

Case Study 22-1

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 22-1** Differentiate lymphomas from leukemias.
- 22-2** Explain the postulated causes of Hodgkin lymphoma.
- 22-3** Describe the morphological and immunophenotypic features of Reed–Sternberg cells as the hallmark for microscopic diagnosis of Hodgkin lymphoma.
- 22-4** Correlate specific Reed–Sternberg cell morphologies with subtypes of Hodgkin lymphoma.
- 22-5** Describe the clinical findings of different subtypes of Hodgkin lymphoma.
- 22-6** List the chromosomal changes seen in individuals with non-Hodgkin lymphoma.
- 22-7** Identify the key features of the non-Hodgkin lymphomas.
- 22-8** Identify the key features of the mature T- and NK-cell lymphomas.
- 22-9** Describe laboratory tests that can resolve differential diagnosis of lymphomas and affect prognosis and treatment options.
- 22-10** Contrast indolent and aggressive non-Hodgkin lymphomas.

Malignant **lymphomas** are a heterogeneous group of cancers that arise from cells of the lymphoid tissue (lymphocytes, or rarely histiocytes or dendritic cells). Whereas leukemias generally arise from the white blood cells in the bone marrow, lymphomas arise from the lymph nodes, spleen, and thymus leading to enlargement of these organs. Lymphomas are broadly divided into two major categories: **Hodgkin lymphoma (HL)** and **non-Hodgkin lymphoma (NHL)**, which differ from each other in clinical, biological, and therapeutic ways.

It is important for students and clinicians to understand the diagnostic challenges often faced by pathologists in evaluating lymphoid proliferations. The distinctions between benign and malignant lymphoid proliferations, Hodgkin lymphoma versus non-Hodgkin lymphoma, and the subcategorization of these entities are all important. Additional tests are often required beyond light microscopy, such as immunological and molecular or cytogenetic assays, which provide relevant diagnostic and prognostic information. This chapter describes

the categories of lymphoma and their important features. Box 22-1 defines the common abbreviations and acronyms used in the chapter.

Hodgkin Lymphoma

Hodgkin lymphoma, formerly called Hodgkin disease before it was proven to be a clonal malignancy, was the first type of lymphoma to be recognized back in 1832.¹ It was not until 70 years later that microscopists described the peculiar cell that is the histologic hallmark of this cancer and that bears the microscopists' names: the **Reed–Sternberg cell** (Fig. 22-1).

Epidemiology, Etiology, and Pathogenesis

Hodgkin lymphoma represents an estimated 6% of all lymphomas in the United States.² It has a bimodal age distribution with younger (20–40 years) or older (>55 years) patients being most affected. The etiology is unknown, though considerable intrigue focuses on possible viral contributions such as **Epstein–Barr virus (EBV)**.³ EBV genomic DNA

BOX 22-1 The Lymphomas: Common Abbreviations and Acronyms

- Anaplastic Large Cell Lymphoma (ALCL)
- Burkitt Lymphoma (BL)
- Classic Hodgkin Lymphoma (CHL)
- Diffuse Large B-Cell Lymphoma (DLBCL)
- Epstein-Barr Virus (EBV)
- Fluorescence In Situ Hybridization (FISH)
- Hairy Cell Leukemia (HCL)
- Hodgkin Lymphoma (HL)
- Mantle Cell Lymphoma (MCL)
- Marginal Zone Lymphoma (MZL)
- Nodular Lymphocyte Predominant Hodgkin Lymphoma (NLPHL)
- Non-Hodgkin Lymphoma (NHL)
- Peripheral T-Cell Lymphoma (PTCL)
- Polymerase Chain Reaction (PCR)
- Rituximab-Cyclophosphamide, Doxorubicin (Hydroxydaunomycin), Vincristine (Oncovin), Prednisone (R-CHOP)
- Small Lymphocytic Lymphoma/Chronic Lymphocytic Leukemia (SLL/CLL)
- World Health Organization (WHO)

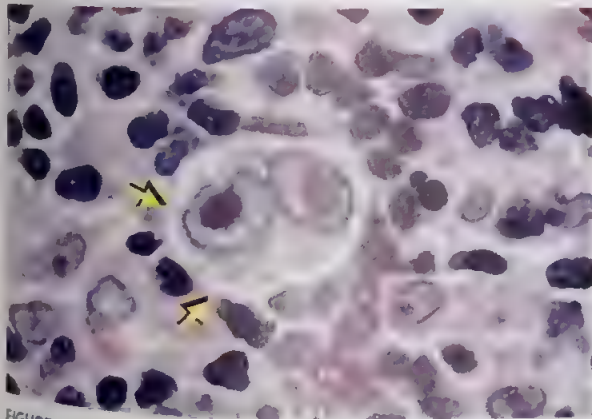


FIGURE 22-1 Reed-Sternberg cell (arrow). Prototypic cell of Hodgkin lymphoma (HL) is characterized by large size, abundant eosinophilic cytoplasm, multinucleation, and inclusion-like macronuclei.

virally encoded latent membrane protein is localized within the malignant Reed-Sternberg cells of approximately 50% of cases, especially those with a preceding form of immunosuppression. Reed-Sternberg cells are defective B lymphocytes with clonal immunoglobulin rearrangements and faulty immunoglobulin transcription.^{4,6} Unlike most other cancers, the malignant cells of HL usually comprise only a small percentage of the total cells in the tumor mass.

Pathology

The cytological hallmark of HL is the presence of an unusual giant cell, the Reed-Sternberg cell. The features of this cell include large size (up to 45 μm in diameter), abundant acidophilic cytoplasm, multinucleated nucleus, and gigantic

nucleoli (see Fig. 22-1). There is often clearing of the chromatin around the nucleoli forming a distinct halo. Identification of Reed-Sternberg cells, however, is not conclusive for diagnosis of HL since similar cells can be seen in a variety of benign and malignant conditions other than HL. The diagnosis often requires immunophenotyping and careful inspection of the cellular, stromal, and clinical setting. True Reed-Sternberg cells are generally surrounded by a mixture of nonneoplastic inflammatory cells composed of small lymphocytes, histiocytes, eosinophils, neutrophils, and plasma cells. The World Health Organization (WHO) subclassifies HL (Table 22-1) into Classic Hodgkin Lymphoma and Nodular Lymphocyte-Predominant Hodgkin Lymphoma based on histologic and, in some cases, immunophenotypic and clinical characteristics.⁷⁻⁸ Many variants of Reed-Sternberg cells have been described (Fig. 22-2A through D).

Nodular Lymphocyte-Predominant Hodgkin Lymphoma (NLPHL)

NLPHL is a relatively uncommon (approximately 10% of cases) but pathologically and clinically distinct variety of HL. The growth pattern is somewhat nodular (Fig. 22-3). The characteristic Reed-Sternberg cells of NLPHL are often referred to as **popcorn cells** (Fig. 22-2B) because of their nuclear lobations and small nucleoli. The surrounding cells are a mixture of small, normal-appearing lymphocytes and histiocytes without many eosinophils or fibrosis. The immunophenotype of the neoplastic Reed-Sternberg cells of NLPHL is similar to germinal center cells (centroblasts)⁴ and distinct from "classical" forms of HL described later in this chapter (Table 22-2). While NLPHL is similar to classic Hodgkin Lymphoma in many ways, the fifth edition of the WHO classification suggests that NLPHL will eventually be removed from the Hodgkin lymphoma category and placed with the mature B-cell neoplasms.⁸

Classic Hodgkin Lymphoma

Classic HL represents the majority of cases of Hodgkin lymphoma cases and is morphologically defined by classic-appearing Reed-Sternberg cells. This entity is further subdivided into four main subtypes based on the cellular and stromal background.

TABLE 22-1 WHO Classification of Hodgkin Lymphoma

Classification	Frequency (%)	Subtype (Frequency %)
Classic Hodgkin Lymphoma	90	Nodular sclerosis (60–80)
		Lymphocyte-rich (5)
		Mixed cellularity (20–25)
		Lymphocyte depletion (<2)
Nodular Lymphocyte-Predominant Hodgkin Lymphoma	10	N/A

Source: World Health Organization Classification of Tumors of Hematopoietic and Lymphoid Tissues, 2016.

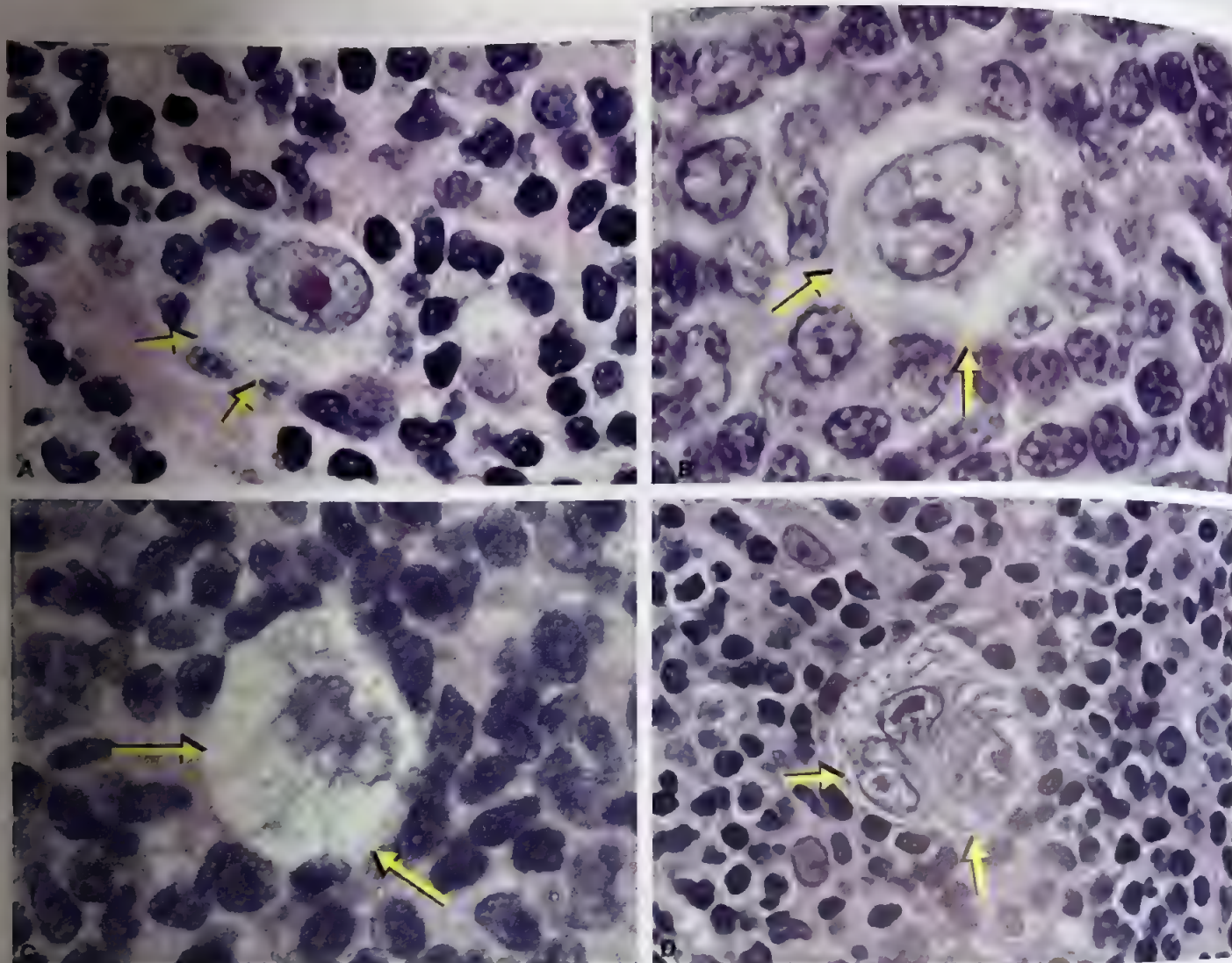


FIGURE 22-2 **A.** Mononuclear Hodgkin cell (arrows). Variant RS cell with single monolobed nucleus and inclusion-like macronucleolus **B.** Nodular lymphocyte predominant Hodgkin lymphoma. Large lymphocyte predominant or popcorn neoplastic cell (arrows): characteristic cell of nodular lymphocyte predominant Hodgkin lymphoma (NLPHL). **C.** Lacunar cell. Large cell with artifactual clear space (lacuna) due to formalin fixation that surrounds the nucleus. Wisps of cytoplasm are visible in the space. **D.** Pleomorphic Reed–Sternberg cell. Large cell with bizarre multinucleated/multilobated nucleus.

Nodular Sclerosis Classic Hodgkin Lymphoma The cardinal histological feature of **nodular sclerosis HL** is nodules of lymphoid tissue rimmed by fibrous bands of collagen (Fig. 22-4A) and a distinctive Reed–Sternberg variant called

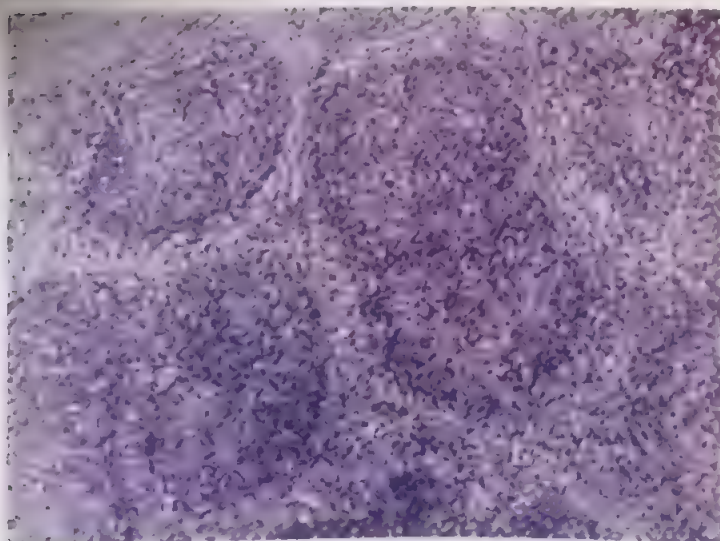


FIGURE 22-3 Nodular lymphocyte predominant Hodgkin lymphoma. Low-power microscopic view showing vaguely nodular pattern.

a **lacunar cell** (Fig. 22-2C). It is so named because it is separated from surrounding cells by a large clear space (a lacuna) located in a sea of lymphocytes, plasma cells, eosinophils, and neutrophils. The lacunar cells may be grouped in clusters, and there may be focal necrosis (Fig. 22-4B).

Mixed Cellularity Classic Hodgkin Lymphoma As its name implies, **mixed cellularity HL** is characterized by Reed–Sternberg cells surrounded by a heterogeneous mixture of cells, including lymphocytes, histiocytes, plasma cells, and eosinophils (Fig. 22-4C). Small areas of necrosis or granulomatous epithelioid histiocytes may be seen. EBV infection of the Reed–Sternberg cells is most frequent in this subtype of HL.

Lymphocyte-Rich Classic Hodgkin Lymphoma A background rich in lymphocytes characterizes **lymphocyte-rich HL**, though the Reed–Sternberg cells exhibit the histologic appearance and immunophenotype of classic Reed–Sternberg cells rather than the popcorn cells seen in NLPHL.

Lymphocyte-Depleted Hodgkin Lymphoma Lymphocyte-depleted Hodgkin lymphoma is the rarest form of classic HL, with more numerous Reed–Sternberg and fewer background cells.

TABLE 22-2 Immunophenotype of Hodgkin (HL) and Non-Hodgkin Lymphomas (NHL)

Antibody	Nodular Lymphocyte-Predominant HL	Classical HL	Diffuse Large B-Cell Lymphoma (NHL)	Anaplastic Large Cell Lymphoma (NHL)
CD30	-	+	-/+	+
CD15	-	+/-	-	-
CD45	+	-	+	+/-
CD20	+	-/+	+	-
CD79a	+	-/+	+	-
Ig	+/-	-	+/-	-
Oct2	+	-/+	+	N/A
Bob1	+	-	+	N/A
EMA	+/-	-	-/+	+/-
ALK	-	-	-	+/-

+ = all cases positive; +/- = majority of cases positive; -/+ = minority of cases positive; - = all cases negative; HL = Hodgkin lymphoma.

Clinical Findings

In the United States, 8,000 new cases of HL are diagnosed each year.⁹ Most affected patients present with nonpainful lymph node swelling. NLPHL has a male predominance, affects younger patients, and is usually localized. Despite excellent long-term survival, patients with NLPHL experience an increased frequency of late relapses, and progression to a large B-cell lymphoma can occur. In contrast, nodular sclerosis classic HL has a female predominance with cervical node enlargement or an anterior mediastinal location. Mixed cellularity HL tends to have widely disseminated disease and symptoms including fever, drenching night sweats, and weight loss. EBV is frequently identified in mixed cellularity and lymphocyte-depleted HL.¹⁰

Staging and Treatment

The diagnosis of HL requires tissue biopsy, microscopic evaluation, and often immunohistochemical stains. Following diagnosis, the patient is "staged" to determine the extent of disease spread, which affects selection of therapy.¹¹ The disease spreads by the lymphatic route to contiguous lymph nodes. Multiagent chemotherapy with or without radiation is the typical mode of treatment. With appropriate therapy, the 10-year survival for stages I and II exceeds 80%, while it approaches 70% in stages III and IV patients receiving more aggressive chemotherapy. Immunotherapy shows promise to further improve outcomes.¹⁰

CRITICAL THINKING QUESTION

22-1 Why can't the presence of Reed–Sternberg cells alone be diagnostic for Hodgkin lymphoma if they are so distinct?
See answers to all Critical Thinking Questions at the back of this book.

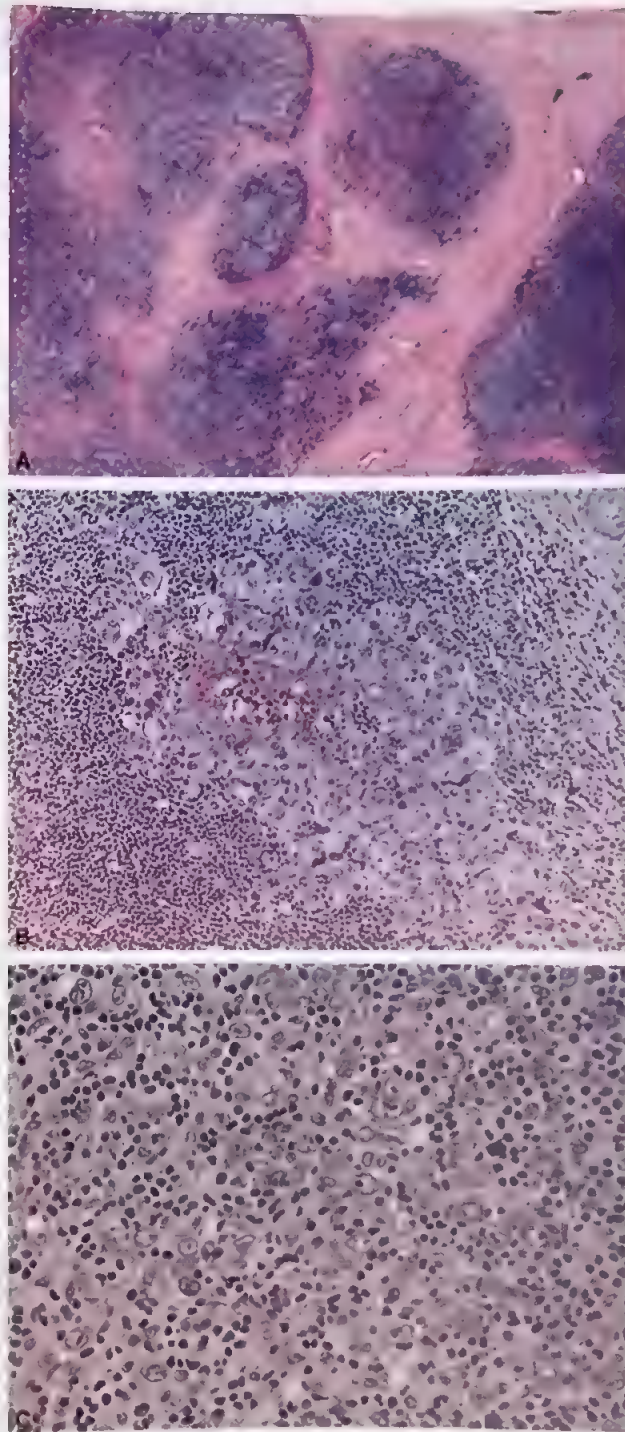


FIGURE 22-4 **A.** Classic Hodgkin lymphoma, Nodular Sclerosis Hodgkin lymphoma (NSHL) subtype. Low-power microscopic view demonstrating nodules of lymphoid tissue rimmed by fibrous cells produced by orderly bands of collagen. **B.** NSHL: Grouped lacunar cells showing central area of necrosis. **C.** Mixed Cellularity Hodgkin lymphoma (MCHL). Easily identified Reed–Sternberg cells and RS variants are seen admixed with small lymphocytes, plasma cells, eosinophils, and histiocytes.

Non-Hodgkin Lymphoma

Epidemiology, Etiology, and Pathogenesis

Non-Hodgkin lymphoma (NHL) is more prevalent than HL at over 77,000 cases per year.⁹ It accounts for 4% of all cancers in the United States. As with all cancers, development of lymphoma is thought to be caused by DNA damage leading to unregulated cell proliferation. The inciting agents in any given patient remain unknown; however, factors such as chemicals, ionizing radiation, viruses (EBV, Human herpesvirus 8, etc.), chronic inflammation, and immunodeficiencies may play a role in initiating and promoting tumor growth.

Numerical or structural alterations of chromosomes are often visible by karyotype.¹² Certain subtypes of NHL have specific chromosomal abnormalities, particularly translocations involving chromosomes 2, 14, or 22 where the **immunoglobulin kappa, heavy chain, or lambda light chain** genes are located, respectively (Table 22-3). These genes are rearranged and transcriptionally active in all B-lymphocytes, which might render B cells prone to structural changes that trigger malignant behavior. Likewise, T-lymphocytes rearrange and express **T-cell receptor** genes, and T-cell lymphomas commonly have a translocation involving the chromosomal sites where these T-cell receptor genes are located.

The translocation partner gene is typically dysregulated to alter its growth-regulating function. An example is Burkitt lymphoma, a B-cell malignancy in which the **MYC** gene located at chromosomal position 8q24 is translocated to the immunoglobulin heavy-chain locus at 14q32 (Fig. 22-5), enhancing cell proliferation driven by dysregulated **MYC** protein. The translocation **t(8;14) IGH-MYC** is also seen in 40% of large-cell lymphomas and therefore is not specific for Burkitt lymphoma. Chromosomal translocation is only one mechanism for oncogene activation. Other mechanisms include gene deletion, mutation, or amplification.

Pathology

Lymphoma classification is based on the histologic growth pattern (for example, nodular or diffuse; Fig. 22-6A and B), cytological features of malignant cells, and immunophenotypic and genotypic features as described in the World Health

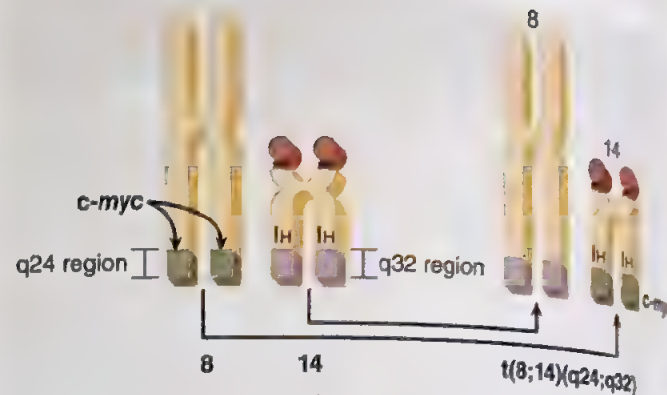


FIGURE 22-5 Reciprocal translocation **t(8;14) (q24;q32)** is seen in majority of cases of Burkitt's lymphoma as well as some non-Burkitt's high-grade lymphomas. A reciprocal translocation of genetic material occurs between chromosomes 8 and 14. A distal portion of the long arm of chromosome 8, containing the **c-myc** oncogene, is translocated to a site adjacent to the immunoglobulin heavy-chain locus on chromosome 14.

Organization classification scheme (Box 22-2).^{2,8} A conceptual understanding requires discussion of the maturation of normal lymphocytes, since each subtype of lymphoma may be thought of as a malignant population of cells arrested at a particular stage of maturation. Thus, each subtype of lymphoma has some attributes of normal lymphoid cells as seen in each of the various normal lymphoid compartments; that is, the precursor (bone marrow), follicular, mantle zone, marginal zone, and interfollicular compartments. As illustrated in Figures 22-7 and 22-8, each of these compartments contains cells with characteristic cytological, immunophenotypic, and genotypic features.

Within lymph nodes, B cells are mostly organized into nodules referred to as follicles. On antigenic stimulation, primary follicles are transformed into secondary follicles containing a germinal center surrounded by a crescent of B cells referred to as the mantle zone. The germinal center is populated with centrocytes (resting B cells), centroblasts (proliferating B cells), dendritic cells, and histiocytes/macrophages. The interfollicular region or paracortex also contains T-lymphocytes and specialized vessels called high endothelial venules which are the point of entry of circulating lymphocytes. Lymphoma may arise from or appear

TABLE 22-3 Common Chromosomal Abnormalities Associated With Malignant Lymphoma

Abnormality	Genetic Loci Involved	Associated Lymphoma
Trisomy 12	-	B-CLL/SLL
Del 13q14	-	B-CLL/SLL
t(11;14)	IGH-CCND1	Mantle cell lymphoma, myeloma
t(14;18)	IGH-BCL2	Follicular lymphoma, diffuse large B-cell lymphoma, double-hit lymphoma
t(8;14)	IGH-MYC	Burkitt lymphoma or diffuse large B-cell lymphoma, double-hit lymphoma
t(2;8)	IGK-MYC	Burkitt lymphoma
t(8;22)	IGL-MYC	Burkitt lymphoma
t(3;14)	IGH-BCL6	Diffuse large B-cell lymphoma, double-hit lymphoma
t(2;5)	NPM1-ALK	Anaplastic large cell lymphoma

B-CLL = B-cell chronic lymphocytic leukemia; SLL = small cell lymphocytic lymphoma.

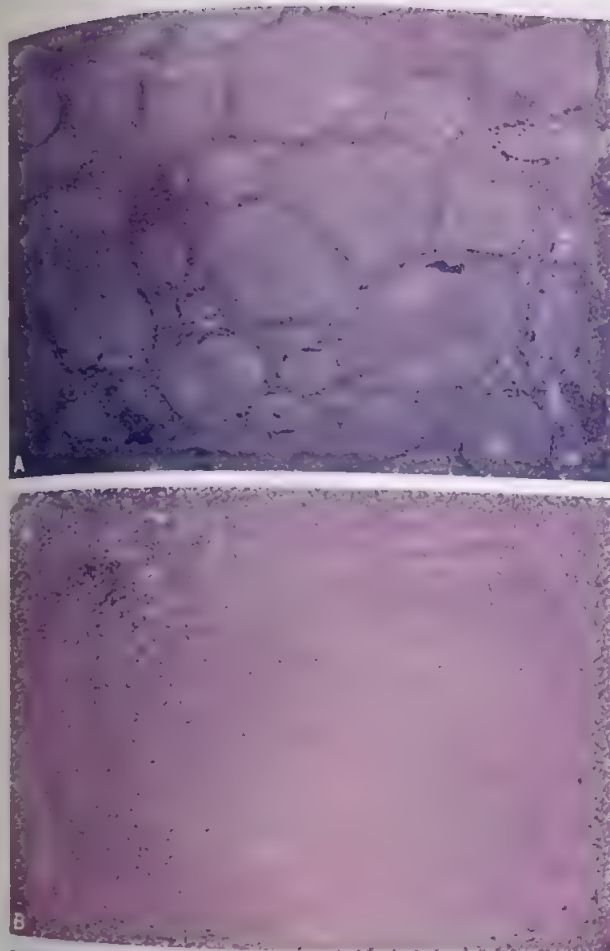


FIGURE 22-6 A. Follicular growth pattern of lymphoma. Neoplastic cells are organized into closely packed nodules (follicles). B. Diffuse growth pattern of lymphoma. Neoplastic cells are arranged in sheet like fashion without follicular organization.

similar to cells in any of these compartments. B-cell lymphomas are by far the most common type of NHL (85% to 90%), while T-cell or natural killer cell lymphomas make up most of the remainder, and true histiocytic or dendritic cell neoplasms are distinctly uncommon (<1%).

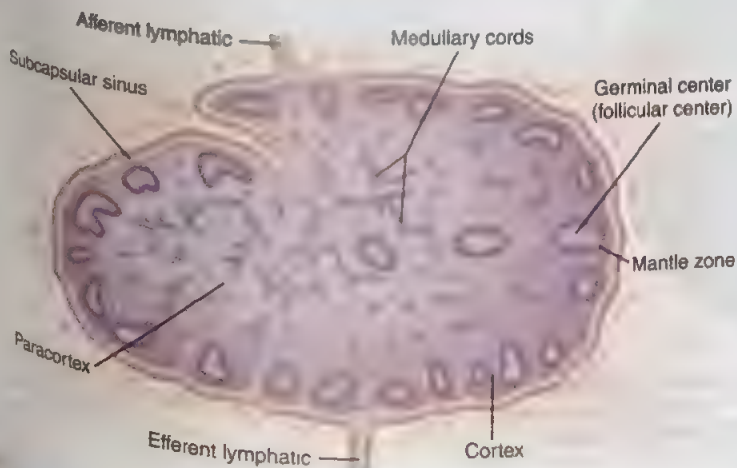


FIGURE 22-7 Anatomical compartments of the lymph nodes.

BOX 22-2 World Health Organization (WHO) Classification of Non-Hodgkin Lymphomas

B-Cell Neoplasms

Precursor B-Cell Neoplasm

- B lymphoblastic leukemia/lymphoma

Mature B-Cell Neoplasms

- Chronic lymphocytic leukemia/small lymphocytic lymphoma
- Lymphoplasmacytic lymphoma
- Splenic marginal zone lymphoma
- Hairy cell leukemia
- Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT-lymphoma)
- Nodal marginal zone B-cell lymphoma
- Follicular lymphoma
- Mantle cell lymphoma
- Diffuse large B-cell lymphoma
- Mediastinal large B-cell lymphoma
- Burkitt lymphoma

T-Cell and NK-Cell Neoplasms

- T lymphoblastic leukemia/lymphoma
- Extranodal NK/T-cell lymphoma, nasal type
- Mycosis fungoides
- Sézary syndrome
- Primary cutaneous anaplastic large cell lymphoma
- Peripheral T-cell lymphoma, unspecified
- Anaplastic large cell lymphoma

Histiocytic and Dendritic-Cell Neoplasms

Note: Uncommon lymphoma subtypes are not listed.

B-Cell Lymphomas

B-cell lymphoma is a malignant proliferation arising from a single B lymphocyte that has lost its ability to control cell proliferation. This B lymphocyte divides repeatedly to form a monoclonal cell population carrying the same genetic defects as the original B cell from which it arose. The cells within a given tumor generally correspond to a stage of B-cell maturation, either a less

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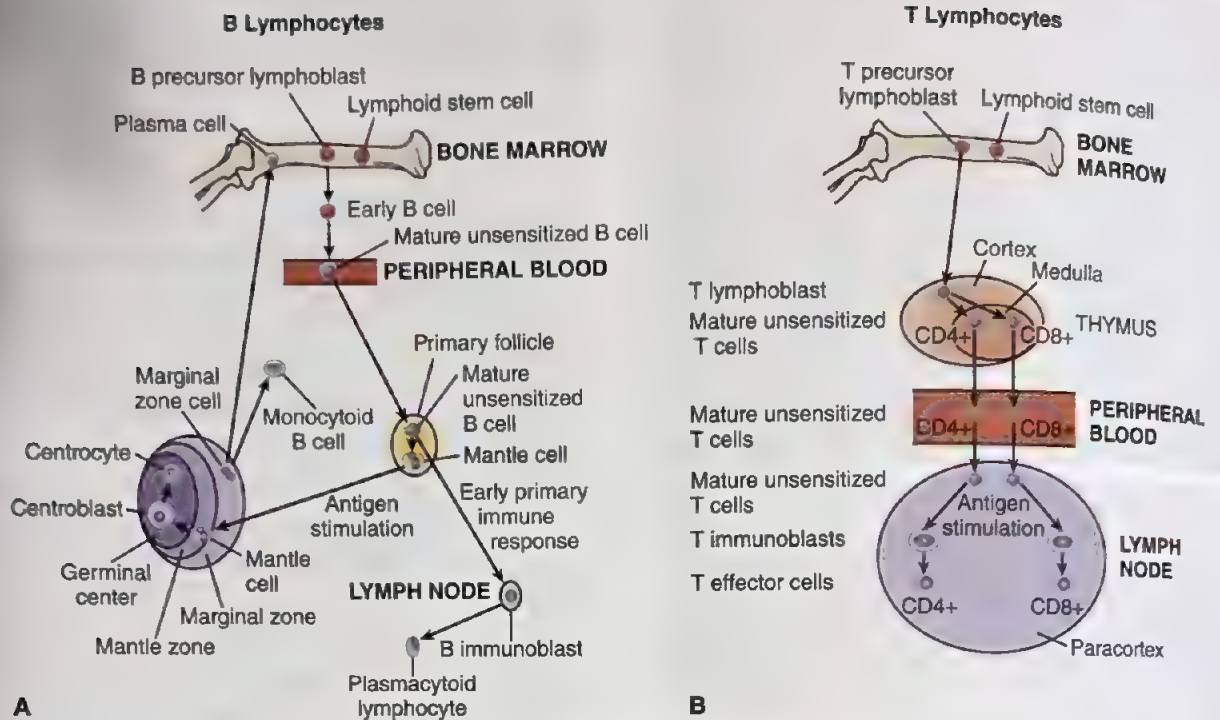


FIGURE 22-8 Maturation of (A) B lymphocytes and (B) T lymphocytes in relation to the pertinent anatomical compartments.

immature naïve B cell, a more mature plasma cell, or somewhere between. Plasma cell myeloma, because of its unique clinical presentation and treatment, is discussed in Chapter 23. B-cell lymphomas predominate in older adults (median age 60 years), except for Burkitt lymphoma and mediastinal large B-cell lymphoma that can occur at younger ages. Within the broad category of B-cell lymphoma, two subtypes (follicular lymphoma and diffuse large B-cell lymphoma) account for the majority of cases (see Fig. 22-9).

Follicular Lymphoma

Follicular lymphoma most commonly affects adults and is usually widespread throughout the body at diagnosis (Table 22-4). Follicular lymphomas exhibit a follicular growth pattern resembling the benign secondary follicles of a reactive lymph node. These follicles are composed of the two cell types found in normal germinal centers, namely centrocytes (cleaved cells) and centroblasts (large noncleaved cells) (Fig. 22-10A and B). Unlike normal germinal centers,

Total Mature NHL = 112,380

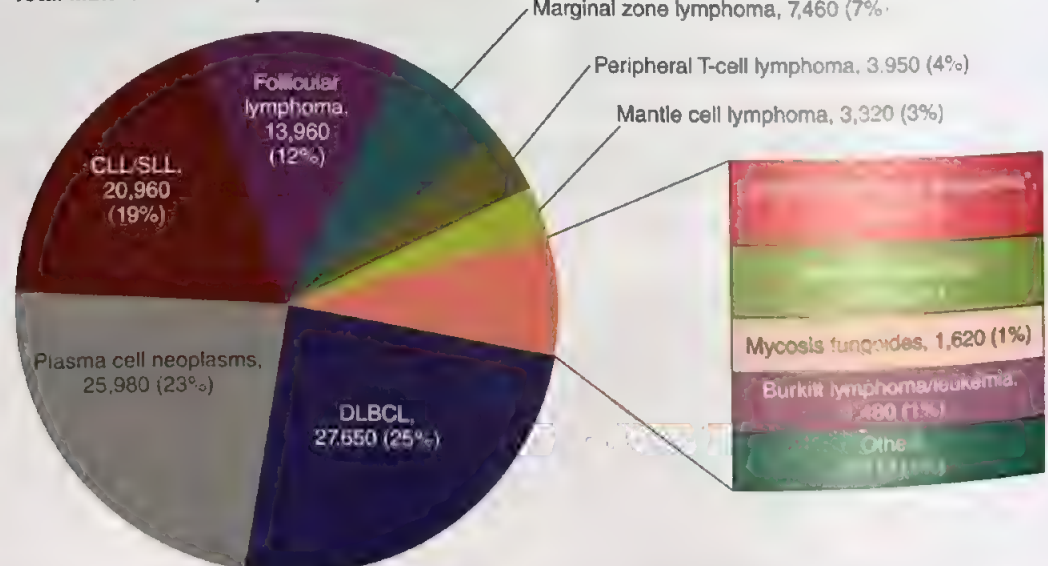


FIGURE 22-9 Estimated Cases and Distribution of Mature Non-Hodgkin Lymphoid Neoplasm Subtypes. United States, 2016. CLL/SLL indicates chronic lymphocytic leukemia/small lymphocytic lymphoma; DLBCL, diffuse large B-cell lymphoma; NHL, non-Hodgkin lymphoma. *Includes Waldenström macroglobulinemia. †Includes Hairy cell leukemia variant. Adapted from Teras LR, DeSantis CE, Cerhan JR, Morton LM, Jemal A, Flowers CR. 2016 US lymphoid malignancy statistics by World Health Organization subtypes. *CA Cancer J Clin*. 2016;66(6):443-459.

TABLE 22-4 Low-Grade B-Cell Lymphomas: Clinical Findings

Lymphoma Subtype	Male: Female	Stage I (%)	Extranodal %	BM (%)	PB (%)	NED at 5 years (%)	Survival Rate at 5 years (%)
B-cell CLL/SLL	2:1	0	Rare	100	>90	<10	10
Mantle cell	3:1	<10	>50	>50	25	0	<40
Follicular center cell	1:1	<10	<50	>50	<15	<25	>60
Lymphoplasmacytic Marginal zone	1.3:1	Rare	Rare	100	<30	0	>70
Extranodal	1:2	>80	100	<15	Rare	>80	100
Nodal	1:2	50?	?	50?	Rare	50?	50

*Pure nodal marginal cell lymphomas are uncommon.

BM = bone marrow involvement; PB = peripheral blood involvement; NED = no evidence of disease.



FIGURE 22-10 A. Architecture of benign (reactive) follicle. Secondary follicle consisting of well-developed mantle zone (1) and germinal center (2) is polarized into light (2) and dark (3) zones. Tingible body macrophages (arrows) are prominent in the dark zone. B. Malignant follicle of follicular lymphoma. Note poorly defined/absent mantle zone and lack of polarization of the germinal center into light and dark zones.

follicular lymphoma nodules tend to lack polarization into light and dark zones, lack tingible-body macrophages, and lack a mantle zone. Centrocytes are small (6 to 15 μm) with scant cytoplasm, irregular nuclei (angulated, clefted), and indistinct nucleoli. Centroblasts are 20 to 40 μm and have a modest amount of cytoplasm, round to oval vesicular nuclei, and small but distinct nucleoli (Table 22-5, Fig. 22-11A and B). Most cases show a predominance of centrocytes (grades 1 and 2), which are resting B cells, which helps explain the indolent clinical behavior of this disease (median survival 6 to 8 years). Progression from follicular lymphoma to a more aggressive, diffuse large-cell lymphoma is common.¹⁷

The immunophenotype of follicular lymphoma is summarized in Table 22-6. At the genetic level, they harbor a unique chromosomal translocation, t(14;18), juxtaposing the *BCL2* gene on chromosome 18 with the immunoglobulin heavy-chain gene on chromosome 14 (see Table 22-3), which causes overexpression of *BCL2* protein and prolonged cell survival.

A recently described entity is *in situ* follicular neoplasia (ISFN), in which germinal centers are partially or completely colonized by clonal B cells carrying the *BCL2* translocation

TABLE 22-5 Indolent B-Cell Lymphomas: Cytological Features of Neoplastic Cells

Lymphoma Subtype	Small Cells	Large Cells
B-cell CLL/SLL	Round (rarely cleaved)	Paraimmunoblasts, prolymphocytes
Mantle cell	Cleaved (rarely round)	Usually absent
Follicular	Cleaved (centrocytes)	Centroblasts
Lymphoplasmacytic	Round	Immunoblasts
Marginal zone	Round, cleaved, and/or monocytoid	Centroblasts, immunoblasts

CLL = chronic lymphocytic leukemia; SLL = small cell lymphocytic lymphoma.

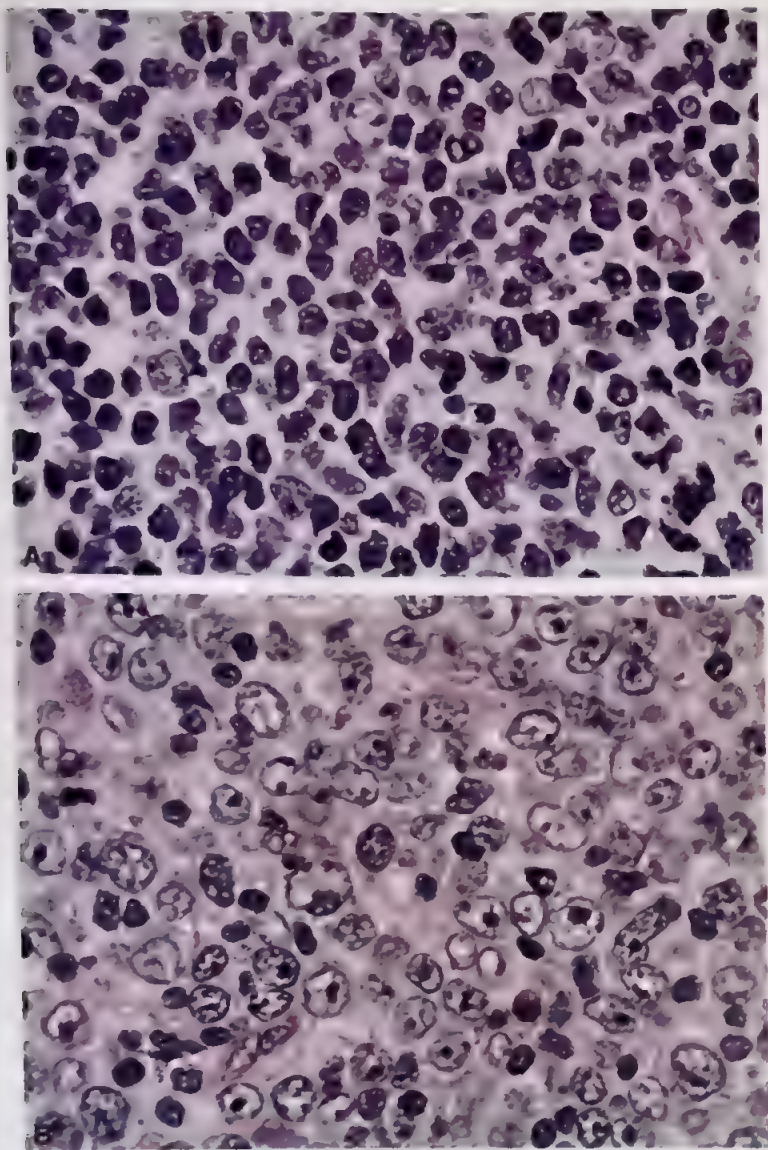


FIGURE 22-11 A. Follicular lymphoma, WHO grade 1. Malignant follicles are characterized by a predominance of centrocytes. B. Follicular lymphoma, WHO grade 3. Malignant follicles are characterized by a predominance of centroblasts.

characteristic of follicular lymphoma. The remaining lymph node is otherwise reactive in appearance. ISFN is identified in 2% of randomly selected lymph node biopsies. The risk of subsequent follicular lymphoma is low (<5%).¹⁴

Mantle Cell Lymphoma

Mantle cell lymphoma (MCL) is a cancer of older adults, often males (see Table 22-4). MCL is composed of clonal B-lymphocytes with the morphological and immunophenotypic features of normal mantle zone cells. The malignant lymphocytes are small- to medium-sized with scant cytoplasm, irregular nuclei, and inconspicuous nucleoli (see Table 22-5, Fig. 22-12A). Most often, MCL has a diffuse or vaguely nodular pattern; however, occasionally a distinct mantle zone pattern is observed surrounding benign (polyclonal) germinal centers.

The MCL immunophenotype shown in Table 22-6 corresponds to the CD5-positive, CD23-negative B cell of normal mantle zone cells. Most cases of MCL demonstrate a unique chromosomal translocation, t(11;14), juxtaposing the *CCND1* gene on chromosome 11 and the immunoglobulin heavy-chain gene on chromosome 14 (see Table 22-3). This translocation results in the overexpression of the cell cycle promoting protein cyclin D1 detectable by immunoperoxidase stain (see Fig. 22-12B). Much less common is a *CCND2* translocation with immunohistochemical expression of the transcription factor SOX11.¹⁵⁻¹⁶

MCL is usually widespread at diagnosis and has a moderately aggressive course (3- to 5-year median survival).¹⁷ The prognosis is better for patients lacking nodal enlargement and having only leukemic involvement.¹⁸

Marginal Zone Lymphoma

Marginal zone lymphoma (MZL) (see Table 22-4) is typically an extranodal clonal proliferation of mucosa-associated lymphoid tissue (MALT-lymphoma) at sites such as stomach.

TABLE 22-6 B-Cell Lymphomas: Immunophenotypic Profile

Lymphoma Subtype	CD5	CD10	CD23	CD25	CD43	SOX11	Cyclin D1	Tdt
B-cell CLL/SLL	+	-	+	-	+	-	-	-
Mantle cell	+	-/+	-	-	+	-	+	-
Follicular center cell	-	+/-	-	-	-	-	-	-
Lymphoplasmacytic	-	-	-	-	+/-	-	-	-
Marginal zone, Extranodal	-	-	-	-	-/+	-	-	-
Marginal zone, Nodal	-	-	-	-	+/-	-	-	-
Marginal zone, Splenic	-/+	-	-	-	-	-	-	-
Hairy cell	-	-/+	-	+	-	+	-	-
Burkitt	-	+	-	-	+/-	-	-	-
B-ALL	-	+	-	-	-	-	-	-

-/+ = less than 50% of cases positive; +/- = more than 50% of cases positive; CLL = chronic lymphocytic leukemia; SLL = small lymphocytic lymphoma.

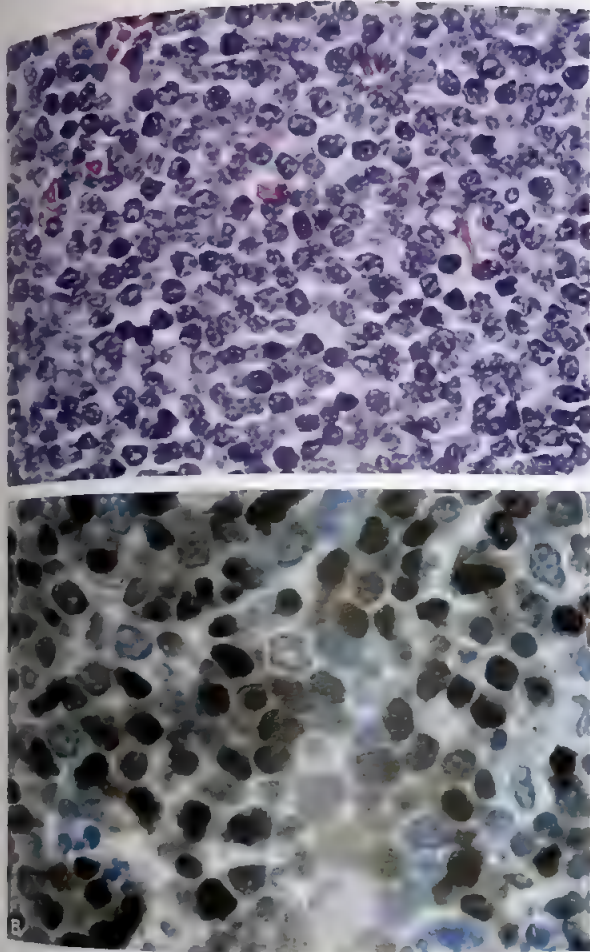


FIGURE 22-12 **A.** Mantle cell lymphoma (MCL). Neoplastic cells are "intermediate" between the small uniform lymphocytes of small lymphocyte lymphoma (SLL) and the centrocytes of follicular lymphoma (FL). **B.** The majority of nuclei of mantle cell lymphoma (MCL) express BCL-1 protein (red-brown reaction product). Immunoperoxidase stain with monoclonal antibody to BCL-1/cyclin D1.

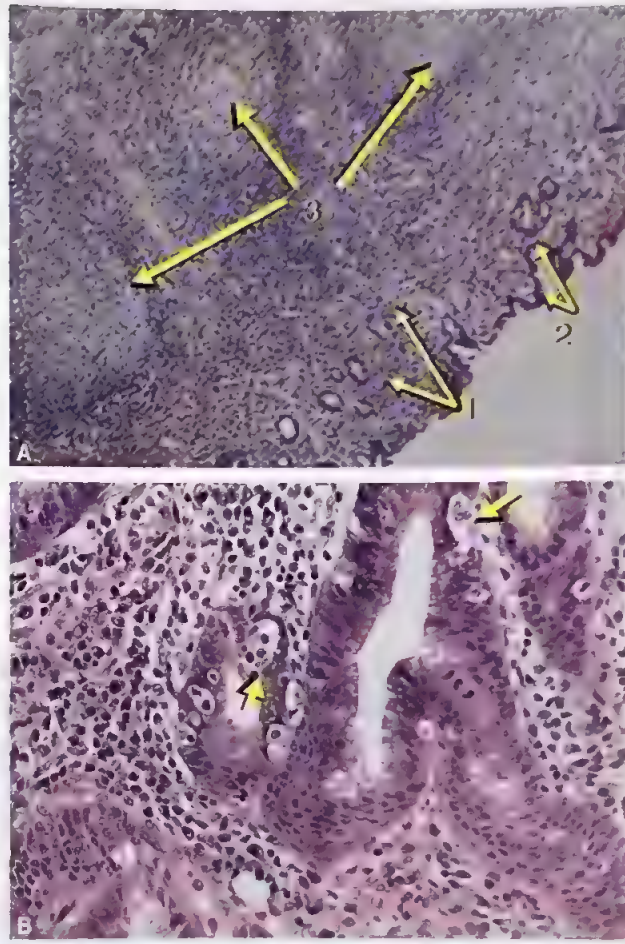


FIGURE 22-13 **A.** Marginal zone lymphoma (MZL). (1) Extranodal clonal proliferation of mucosa-associated lymphoid tissue (MALT) lymphoma of the stomach. (2) MALT lymphoma infiltrates the gastric mucosa and extends into gastric epithelium. (3) Reactive follicles are commonly present. **B.** Characteristic lymphoepithelial lesions (arrows) of gastric MALT lymphoma are composed of clusters of small, atypical lymphocytes (CD20 positive B cells) within the glandular epithelium.

salivary gland, lung, thyroid, or orbit.¹⁹ The marginal zone of a normal lymph node is a poorly defined anatomical compartment.²⁰ However, in other lymphoid tissues (i.e., spleen, Peyer's patches of the intestine), the marginal zone is better defined²¹ and is filled with small, cleaved lymphocytes with more abundant pale-staining cytoplasm than a centrocyte. Marginal zone cells can undergo differentiation into monocytoid B cells (resembling monocytes) or into plasma cells (see Table 22-5). The characteristic immunophenotype of MZL is shown in Table 22-6.

Extranodal MZL arises under conditions of chronic antigenic stimulation (e.g., longstanding infection).²² An example is *Helicobacter pylori* gastritis. Importantly, the tumor might resolve if the stimulus is removed. For example, marginal zone lymphoma of the stomach can be treated by eradicating the offending bacterial infection (*H. pylori*).²³

The histologic features of extranodal MZL (MALT lymphoma) are highly characteristic (Fig. 22-13A and B). Adjacent to the epithelium of involved organs are reactive follicles

and collections of neoplastic small lymphocytes, monocytoid B cells, and plasma cells. A constant feature is infiltration of epithelium by neoplastic lymphocytes, resulting in the formation of lymphoepithelial lesions.

A second pattern of MZL is lymph node-based and is referred to as nodal MZL. Nodal MZL is frequently observed in patients with longstanding chronic inflammation due to autoimmune disorders such as Sjögren's syndrome or Hashimoto's thyroiditis. Histologically, the paracortex and sinuses are filled with a monoclonal population of small lymphocytes and monocytoid B cells.

A third category is splenic MZL, where the spleen exhibits nodular expansion of the white pulp by neoplastic marginal zone cells (Fig. 22-14). Patients characteristically have massive splenomegaly with minimal adenopathy. Bone marrow and peripheral blood involvement by neoplastic villous lymphocytes is common. The clinical course is indolent, and necromy may result in long remission.



FIGURE 22-14 Splenic marginal zone B-cell lymphoma. White pulp nodules (1) show expansion of the marginal zone (2) by a population of small monoclonal B cells which express the phenotype of splenic marginal zone cells. Neoplastic cells infiltrate the red pulp (3).

Small Lymphocytic Lymphoma/Chronic Lymphocytic Leukemia

Small lymphocytic lymphoma (SLL) is the tissue equivalent of chronic lymphocytic leukemia (CLL) in the marrow and blood. The growth pattern of SLL is diffuse, although pseudo-follicular growth centers may be observed (Fig. 22-15A). In the early phase of nodal involvement, the neoplastic cells are confined to the interfollicular areas, and later there is total effacement of lymph node architecture. The majority of the neoplastic cells appear cytologically similar to normal small lymphocytes (see Fig. 22-15B), while the growth centers also have large lymphocytes referred to as *prolymphocytes*. SLL may show evidence of plasmacytoid differentiation with the presence of clonal cytoplasmic immunoglobulin. The immunophenotype of SLL is shown in Table 22-6.

Most SLL/CLL exhibit clonal genetic abnormalities detected by a panel of fluorescence in situ hybridization (FISH) assays. Commonly detected are del 13q14 (50%) or trisomy 12 (20%). A poor prognosis is conferred if there

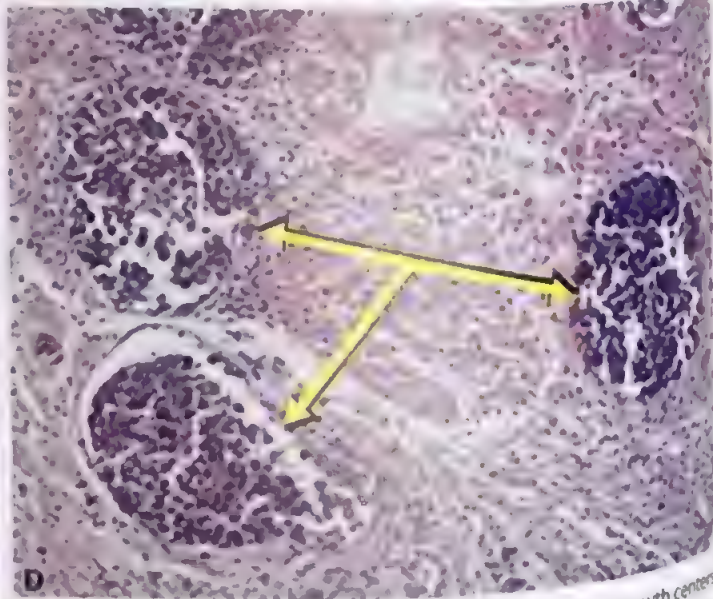
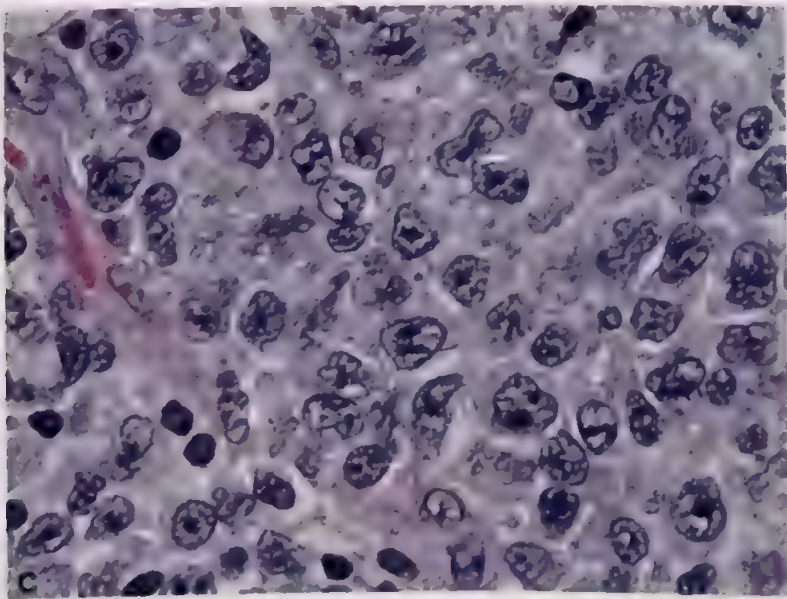
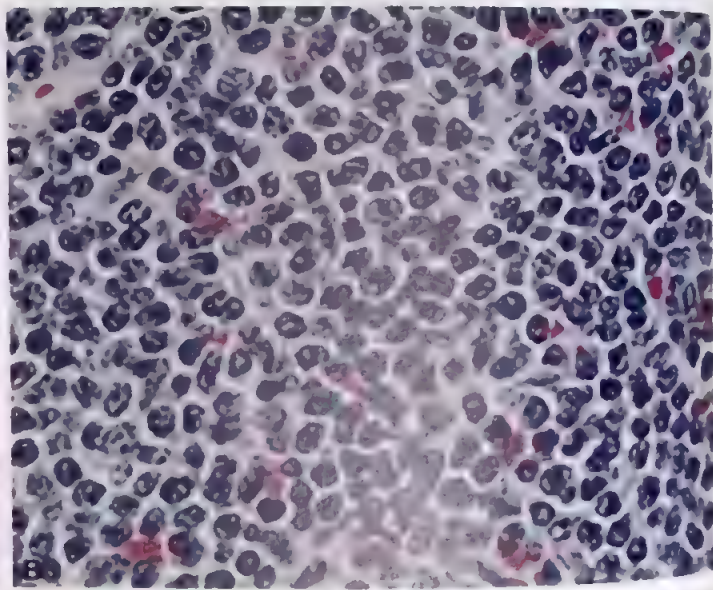


FIGURE 22-15 **A.** Small lymphocytic lymphoma. Diffuse effacement of lymph node by proliferation of small lymphocytes. Pale areas are growth centers (arrows). **B.** Cytological detail of small lymphocytes of SLL. Cells have scant cytoplasm with uniform nuclei, coarsely clumped chromatin, and indistinct nucleoli. **C.** Diffuse large B-cell lymphoma (DLBCL). This common lymphoma is composed of a mixture of large B cells that exhibit centroblastic, immunoblastic, and pleomorphic morphology. **D.** Intravascular large cell lymphoma. Small vessels (capillaries) are occluded by atypical large cells (arrows) with extension into the parenchyma of the involved organ.

is deletion of chromosome 17p13 where the *TP53* gene is located or del 11q22 (*ATM* gene loss). Hypermutation of the immunoglobulin gene variable region correlates with improved survival.²⁴

SLL/CLL is a cancer of older adults and is usually wide spread at diagnosis. The disease is indolent and can be managed with chemotherapy. Transformation to a more aggressive lymphoma can occur, so-called Richter's transformation.²⁵

Diffuse Large B-Cell Lymphoma

Diffuse large B-cell lymphoma (DLBCL) represents the most frequently diagnosed category of NHL worldwide, and it usually affects older adults. DLBCL exhibits a diffuse growth pattern and is composed of clonal large B lymphocytes (Fig. 22-15C). It is subdivided into two major subtypes reflecting its cell of origin: **germinal center (GCB)** or **activated B cell (ABC, or non-GCB)**.²⁹ Patients with GCB type have better overall survival. Gene expression profiling of RNA or protein is typically used to distinguish the two subgroups, most commonly using a panel of immunohistochemical assays and an algorithm called the **Hans criteria**.³⁰

Chromosomal translocation of *MYC*, *BCL2*, and/or *BCL6* have prognostic implications. Approximately 10% of DLBCLs have *MYC* gene rearrangement, and of those, approximately half also have *BCL2* and/or *BCL6* translocation (so-called double-hit lymphomas) with inferior survival.³¹

Most cases of DLBCL arise de novo; however, transformation from a lower grade B-cell lymphoma (SLL, MCL, MZL, follicular lymphoma, or NLPHL) may instead give rise to the tumor, in which case, genotype or immunophenotype may provide clues to the predecessor (Table 22-6). DLBCL is an aggressive lymphoma if left untreated; however, cure is possible with multiagent chemotherapy.³²

Lymphoplasmacytic Lymphoma

Lymphoplasmacytic lymphoma is a cancer of older adults in which the neoplastic lymphocytes exhibit plasmacytoid features and express monoclonal surface and cytoplasmic immunoglobulin, usually of the IgM class. Immunophenotype is shown in Table 22-6. The characteristic *MYD88* L265P mutation can be detected by PCR or sequencing (see Table 22-3).²⁶ Clinically, there is an association with **Waldenström's macroglobulinemia**, which means the presence of an IgM monoclonal gammopathy that can cause hyperviscosity of the blood.²⁷ (See Chapter 23.) This disease is typically indolent, but large cell lymphoma transformation may occur. Figure 22-15D shows an intravascular large cell lymphoma with occluded capillaries.

Hairy Cell Leukemia

Hairy cell leukemia (HCL) is a rare indolent neoplasm of mature lymphocytes with oval to kidney-shaped nuclei and abundant cytoplasm and "hairy" cell surface projections. HCL involves the peripheral blood, bone marrow, and splenic red pulp. Patients present with splenomegaly, pancytopenia, circulating hairy cells, and often, monocytopenia. The immunophenotype is shown in Table 22-6. Virtually all cases harbor a *BRAF* V600E mutation leading to constitutive activation of the pathway driving neoplastic cell proliferation.²⁸

Burkitt Lymphoma

Burkitt lymphoma (BL) is endemic to Africa and also accounts for approximately one-third of non-African pediatric lymphomas (sporadic BL) and a high percentage of lymphomas in immunocompromised patients. The uniform neoplastic cells are medium-sized lymphocytes with multiple small nucleoli and intensely basophilic cytoplasm (Fig. 22-16A) that contain lipid vacuoles. The proliferation rate is among the highest of any cancer (see Fig. 22-16B), with an estimated doubling time of 1 day, which ironically could render it more susceptible than slower growing lymphomas to a chemotherapeutic cure. Programmed cell death (apoptosis) is also very high, leading to a starry sky pattern of tingible-body macrophages in tumor tissue as these phagocytic cells clear out apoptotic debris. A tingible-body macrophage is a prominent macrophage with irregular cellular debris. Figure 22-16C shows a young patient with Burkitt's lymphoma before and after treatment.

As previously discussed, Burkitt lymphoma has t(8;14) *IGH-MYC*, and many cases also harbor the EBV genome. The immunophenotype is shown in Table 22-6.³³

B-Lymphoblastic Leukemia/Lymphoma

B-lymphoblastic leukemia/lymphoma is a cancer of precursor B lymphocytes, most commonly affecting young children. B-lymphoblastic lymphoma (B-LBL) is the tissue-based (nodal or extranodal) equivalent of B-cell acute lymphoblastic leukemia (B-ALL) seen in bone marrow and blood (Fig. 22-17).³⁴ The lymphoblasts have finely dispersed chromatin with indistinct nucleoli. Mitotic figures are numerous. The immunophenotype is shown in Table 22-6.

T-Cell and Natural Killer (NK)-Cell Lymphomas

Because of phenotypic and clinical similarities, the **mature T- and NK-cell lymphomas** are grouped together in the WHO classification. Although relatively uncommon, they are among the most aggressive of hematolymphoid malignancies. NK-lymphomas demonstrate a predilection to involve certain demographic groups, particularly Asians and Native Americans.

Mature T-cell lymphomas correspond to a post-thymic stage of T-cell differentiation. Most are of the T_H type, which differentiate into either CD4-positive "helper" T cells or CD8-positive "cytotoxic" T cells. A smaller set are of the T_H type or NK cells that lack a T-cell receptor complex (CD3 negative) but otherwise are similar to cytotoxic T cells.

Peripheral T-Cell Lymphoma (PTCL), Unspecified

Most mature T-cell lymphomas are composed of a polymorphous mixture of small, medium, and large T lymphocytes. Benign eosinophils, plasma cells, and epithelioid histiocytes are often admixed with the neoplastic cells, and high endothelial venules may be prominent (Fig. 22-18). Nodal location is most common; however, extranodal (skin, subcutaneous tissue, spleen, viscera, bone marrow) sites are often involved in advanced stages. Involved lymph nodes demonstrate either diffuse effacement of nodal architecture or an interfollicular growth pattern. Immunophenotypically, this group of lymphomas usually exhibits a mature (post-thymic) T-cell phenotype

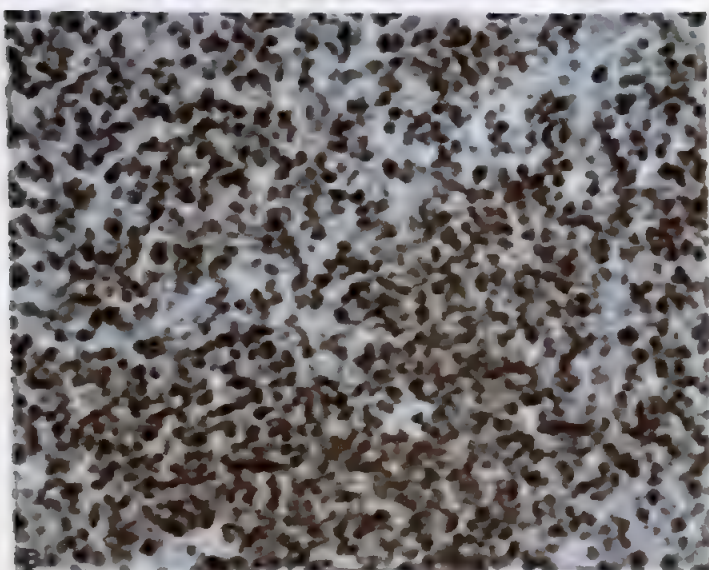
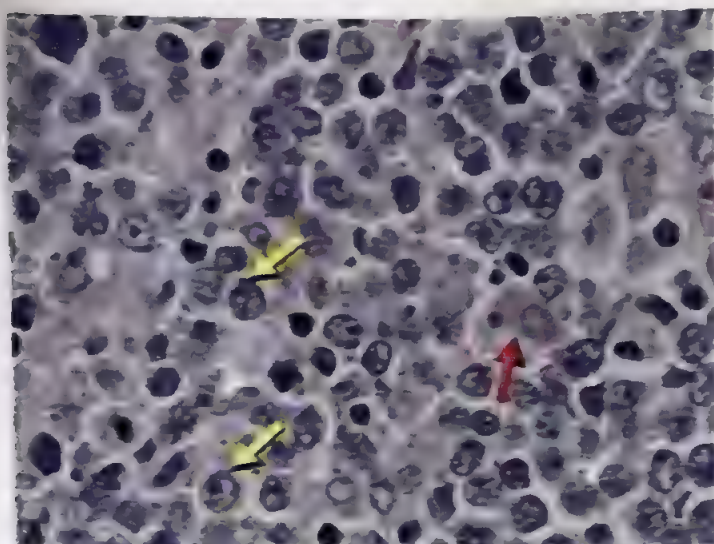


FIGURE 22-16 A. Burkitt lymphoma. Small uniform cells (yellow arrows with moderate amount of cytoplasm, round nuclei and multiple small nuclei are admixed with cellular debris and tingible body macrophages (red arrow). B. High proliferation rate of Burkitt lymphoma detected by high percentage of nuclei showing positive staining for MIB-1 Ki-1 (red brown reaction product). Immunoperoxidase stain with monoclonal antibody to the proliferation antigen MIB-1 Ki-1. C. Burkitt's lymphoma before (left) and after treatment (right).

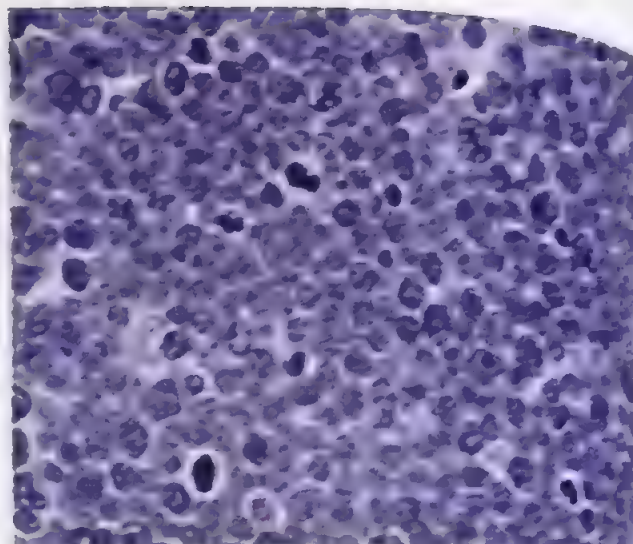


FIGURE 22-17 Precursor B lymphoblastic leukemia/lymphoma. Cells have scant cytoplasm, finely dispersed ("blastic") chromatin, and mitotic activity.

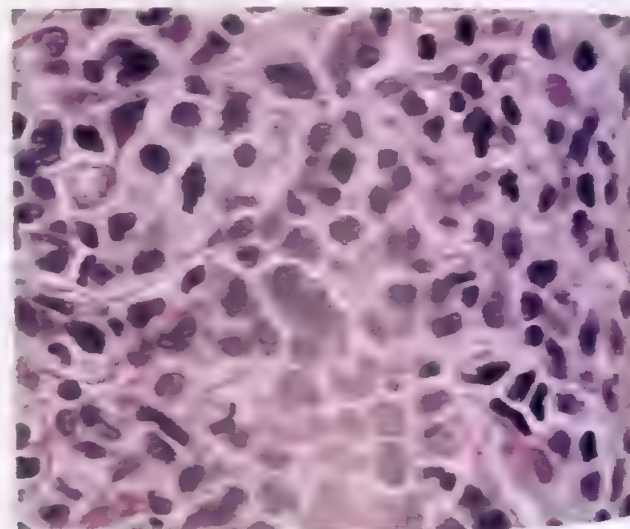


FIGURE 22-18 Peripheria T-cell lymphoma (PTCL). Unspecified PTCL usually consists of a mixture of mature-appearing lymphoid cells which are small in size giving a polymorphous appearance.

(TdT-, CD3-, CD4-, CD8-) and abnormal loss of CD2 and CD7 is common. PTCL is a progressive cancer most often in adults, and overall 5-year survival is only 20%.

Anaplastic Large Cell Lymphoma (ALCL) ALK-positive and ALK-negative ALCL

Anaplastic large cell lymphoma (ALCL) is a distinct clinical entity of young patients (Fig. 22-19A). It is a large neoplastic lymphoma with a characteristic face of an eosinophilic inclusion-like region adjacent to the nucleus (Fig. 22-19B). By immunohistochemistry, there is a loss of the normal Golgi pattern of CD30 positivity, and the presence or absence of ALK expression defines two major subtypes. ALK-positive ALCLs have a characteristic translocation (2;5) NPM1-ALK altering the location of ALK protein. This is usually absent, though the cells are T cells based on rearrangement of the T-cell receptor in greater than 50% of cases.

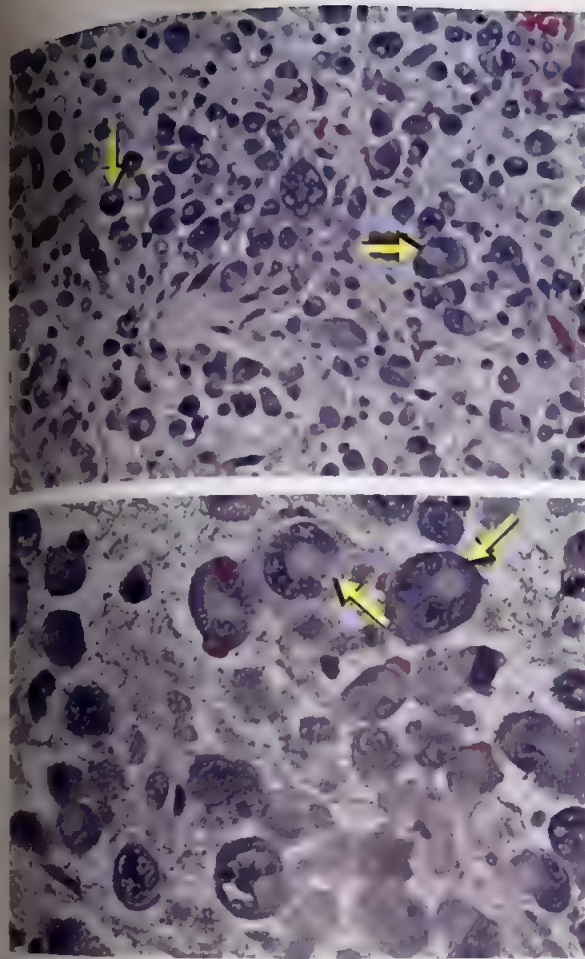


FIGURE 22-19 A. Systemic anaplastic large cell lymphoma (ALCL). Pleomorphic cells that vary in size and shape. "Hallmark" cells are readily apparent (arrows). **B.** Detailed view of several "hallmark" cells (arrows) showing kidney- or horseshoe-shaped nuclei and juxtanuclear eosinophilic inclusion-like zone.

cases. Compared with most other T-cell lymphomas, ALCL is unusual in that it is generally responsive to therapy. Overall survival is approximately 80% at 5 years.³⁶

Breast-implant associated ALCL is a provisional entity in the current WHO classification that is a form of ALK-negative ALCL arising in the association with a breast implant. Most patients have excellent outcomes after removal of the implant.³⁷

Primary Cutaneous T-Cell Lymphomas

Primary involvement of the skin by lymphoma is usually of the T-cell type. **Mycosis fungoides** is most common, sometimes accompanied by **Sézary syndrome**.³⁸ Other CD30-expressing T-cell neoplasms of the skin include lymphomatoid papulosis³⁹ (indolent but can transform to aggressive lymphoma) and primary cutaneous anaplastic large-cell lymphoma⁴⁰ (less aggressive than the noncutaneous version).

Mycosis fungoides and Sézary syndrome have malignant T cells with a peculiar cerebriform nucleus. In 90% of cases, these cells exhibit a mature "helper" phenotype (pan-T+, CD4+, CD8-) and in 10% a cytotoxic phenotype

(pan-T+, CD4-, CD8+). The tumor progresses slowly over years to decades from cutaneous patches to thickened cutaneous plaques (Fig. 22-20A) and tumor nodules produced by massive local infiltrates of the skin by the characteristic cerebriform cells of mycosis fungoides (Fig. 22-20B). In late stages, systemic dissemination to the lymph node, blood, and visceral organs occur and transformation to a large cell lymphoma. The prognosis for mycosis fungoides confined to the skin is relatively good, with a median survival of greater than 10 years. Extracutaneous spread, however, is associated with a median survival of less than 1 year.

Sézary syndrome is characterized by the triad of erythroderma, generalized lymphadenopathy, and clonally cerebriform T cells (Sézary cells) in skin, lymph nodes, and blood (Fig. 22-21). Sézary syndrome is an aggressive disease with a 5-year overall survival rate of 10% to 30%.

CRITICAL THINKING QUESTION

22-2 Among all the chromosomal abnormalities seen in the different non-Hodgkin lymphomas, what is the key defect causing the condition?

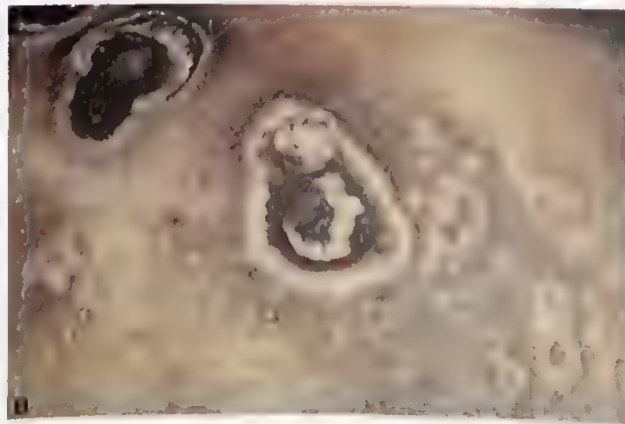
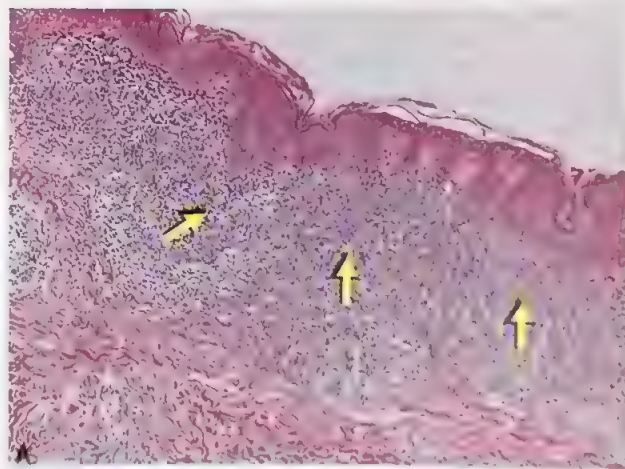


FIGURE 22-20 A. Mycosis fungoides. Plaque lesion of MF showing band like infiltrate (arrows) of dermis by atypical lymphocytes that extend into the overlying epidermis. **B.** Mycosis fungoides, tumor stage. Tumor nodules are produced by massive local infiltrates of the skin by the characteristic cerebriform cells of mycosis fungoides.

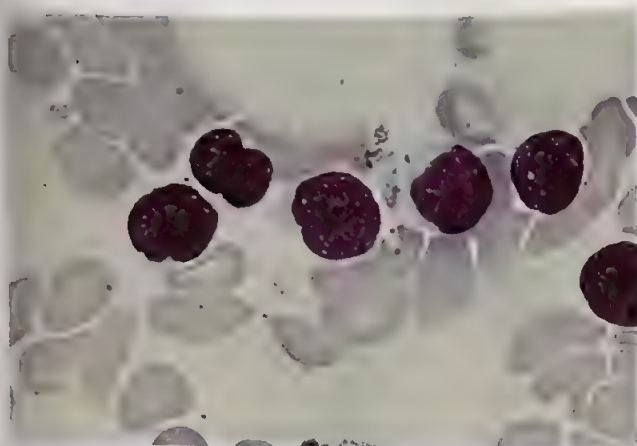


FIGURE 22-21 Sézary cells in the peripheral blood from a patient with Sézary syndrome.

T-Lymphoblastic Leukemia/Lymphoma

T-lymphoblastic leukemia/lymphoma is a neoplasm of lymphoblasts, with the T-LBL component forming a mass in the thymus, mediastinum, or nodal or extranodal sites. The lymphoblasts express TdT and CD3 considered to be specific for T lineage. Non-T-cell antigens may also be expressed, such as CD79a, CD13, and CD33. The T-cell receptor (TCR) gene is clonally rearranged. Involved tissues have complete effacement by a monotonous population of mitotically active blasts. Prompt diagnosis and aggressive therapy is required.³⁴

ADVANCED CONTENT

Other T/NK Mature Lymphomas

The mature T/NK disorders that primarily present in extranodal sites or as disseminated/leukemic disease are distinct yet rare disorders. The interested reader is directed to the specific references for more detailed information.⁴¹⁻⁴⁵

Histiocytic and Dendritic Cell Tumors

The vast majority of lymphomas are derived from lymphocytes. Rare cases present clinically and appear microscopically as lymphoma; however, at the immunological and genetic level they are not lymphoid; rather, they are antigen presenting accessory cells (dendritic cells) or phagocytic cells (histiocytes).⁴⁶

Diagnostic Evaluation of Lymphoid Neoplasia

Tissue biopsy is required for the diagnosis and categorization of lymphomas and lymphoproliferative disorders. The mainstay of diagnosis is histologic evaluation of well-stained tissue sections by an experienced pathologist. Ancillary studies are often required, including immunophenotypic studies by immunohistochemistry or flow cytometry, cytogenetics by karyotype or fluorescent in situ hybridization (FISH), and nucleic acid tests such as polymerase chain reaction (PCR) or sequencing. Careful handling of biopsy tissue is required to ensure that adequate material is triaged for diagnostic

tests. Guidelines for the proper handling and processing of lymphoid tissue are available.⁴⁷ The diagnostic difficulties fall into a small number of categories; namely, differentiating (1) benign *versus* malignant lymphoproliferations, (2) lymphoma *versus* nonlymphoid lineage, (3) T-cell *versus* B-cell lineage, (4) HL *versus* NHL, and (5) subclassifying tumor type.

Benign Versus Malignant

If light microscopic evaluation fails to distinguish a benign from a malignant lymphoproliferation, demonstrating immunophenotypic or genotypic monoclonality favors a neoplastic diagnosis. For B cells, immunophenotypic monoclonality is defined as restriction of immunoglobulin light-chain production by a population of cells to a single light-chain class, either kappa (κ) or lambda (λ). Operationally, light-chain monoclonality is present if the percentage of kappa-positive cells to lambda-positive cells (kappa-to-lambda ratio) falls outside of the expected ("normal") value (2:1 kappa to lambda). An example of immunophenotypic monoclonality demonstrated by flow cytometry is illustrated in Figure 22-22.

Evidence for T-cell malignancy usually relies on immunophenotypically demonstrating an aberrant T-cell phenotype. Benign T-cell proliferations generally express a "normal" T-cell phenotype in which all pan-T-cell antigens (CD2, CD3, CD5, CD7) are expressed by individual T cells. On the other hand, in most T-cell malignancies, one or more of the pan-T-cell antigens are not expressed, with CD5 and CD7 being the most frequently absent antigens.

A genotypic definition of B- and T-cell clonality is feasible based on clonal rearrangement of the T-cell receptor or immunoglobulin genes detectable by PCR or sequencing (see Chapter 35). T-cell receptor or immunoglobulin gene rearrangement is generally useful in assigning T- versus B-cell lineage in a neoplasm. Every neoplasm also has clonal gene variants such as translocation or deletion detectable by cytogenetics (karyotype, FISH) or by molecular tests that also can detect mutation or small insertion or deletion (PCR, sequencing). The identification of a clonal genetic feature is

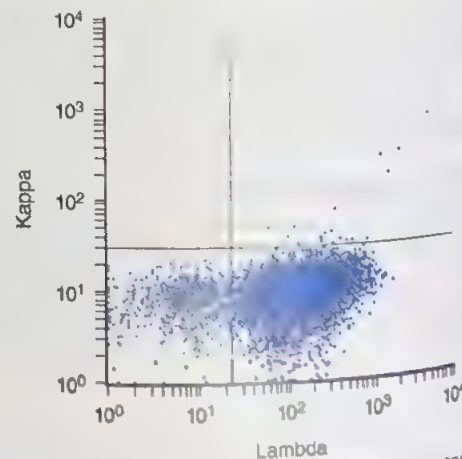


FIGURE 22-22 B-cell monoclonality detected by flow cytometry. The dense cluster of events in the lower right quadrant indicates a monoclonal λ population.

presumptive evidence of a neoplasm rather than a reactive lymphoproliferation.

Although karyotypic analysis in a search for nonrandom chromosome abnormalities might be useful in distinguishing benign from malignant lymphoproliferations or subcategorizing a neoplasm, mature lymphocytic malignancies may fail to grow and divide in cell culture, whereas benign cells in a sample might grow, leading to false negative results. FISH, PCR, and sequencing have emerged as powerful techniques for demonstrating diagnostically important genetic features and quantifying residual tumor after treatment (Fig. 22-23).

Lymphoma Versus Non-Lymphoma

The differential diagnoses of large-cell lymphoma and non-lymphoid malignancies, such as amelanotic melanoma, poorly differentiated carcinoma, germ cell neoplasms, and round cell sarcomas, are a frequently occurring problem in surgical pathology. Differential diagnosis is facilitated by determining the immunophenotype of tumor cells. This is most commonly done by staining the cells using a panel of antibodies directed against cell type-specific antigens. Although no single antibody is either 100% specific or sensitive for identifying the "cell of origin" of a tumor, with the aid of a panel of antibodies it is generally possible to define a phenotypic profile that reveals tumor type. From a therapeutic perspective it is most important to resolve the differential—carcinoma versus lymphoma versus sarcoma versus melanoma—which can usually be accomplished by a relatively small antibody panel (Table 22-7). Additional refinement of the diagnosis is possible with larger panels.

T-Cell Versus B-Cell Lymphoma

Although morphological features may suggest a B-cell or T-cell phenotype, ancillary tests are required for specific distinction between B-cell and T-cell lymphoma. The immunophenotypic demonstration of light-chain monoclonality and the expression of B-lineage antigens (CD19, CD20, CD22), or of an aberrant B-cell phenotype by the cells of a malignant lymphoma, are indicative of a B-cell lymphoma. Similarly,

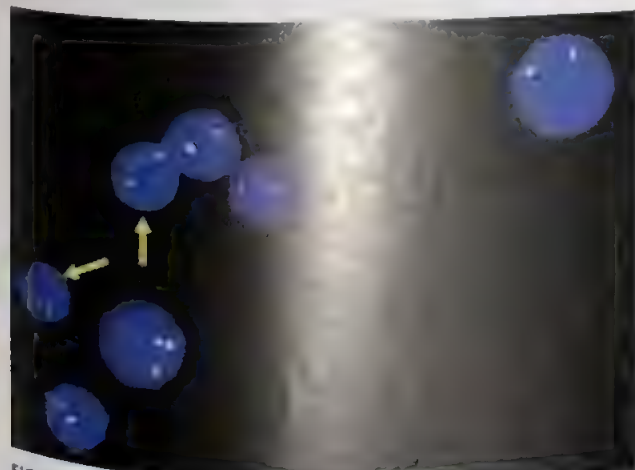


FIGURE 22-23 Fluorescence in situ hybridization (FISH) analysis of B-cell lymphoma using two color break apart probe. Arrowed cells show one normal fusion signal and two abnormal break-apart signals indicating breakage within the IgH (heavy chain) gene switch region

TABLE 22-7 Antibody Panel for Distinguishing the Four Major Categories of Cancer

Cancer Type	Antibody			
	Cytokeratin	Vimentin	CD45	S100
Carcinoma	+	-	-	-
Sarcoma	-	+	-	-
Lymphoma	-	±	+	-
Melanoma	-	±	-	+

the expression of a normal or abnormal T-cell phenotype by the malignant cells is associated with T-cell malignancy. Gene rearrangement studies appear to be more sensitive than immunophenotypic studies in distinguishing T-cell versus B-cell lymphoma; however, relatively few lymphomas require this technically more demanding procedure to make this distinction.

Hodgkin Lymphoma (HL) Versus Non-Hodgkin Lymphoma (NHL)

On morphological grounds alone, it can be difficult to distinguish HL from NHL. For this reason, as well as important therapeutic implications, there has been considerable interest in the utility of ancillary techniques. One immunophenotypic distinction between these two cancers is the presence of light-chain monoclonality in B-cell NHLs and its absence in HL.⁴⁹

The differential diagnosis of HL includes certain NHLs (PTCL, ALCL, etc.) and various benign conditions such as infectious mononucleosis. Careful attention to morphological features in association with immunohistochemical analysis usually resolves the dilemma. The neoplastic cells of all subtypes of CHL are CD45 and *ALK* protein-negative, CD30-positive, and usually CD15-positive (Fig. 22-24). They are most often negative for pan-B and T-cell antigens (Table 22-2). PTCL and

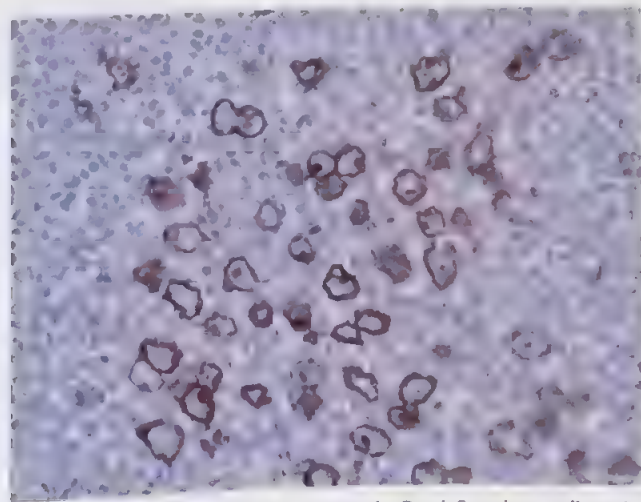


FIGURE 22-24 Expression of CD30 antigen by Reed-Sternberg cells and variants in CHL (red-brown reaction product). Three patterns of staining are present—cytoplasmic membrane, diffuse cytoplasmic, and juxta nuclear dot ("Golgi"). Immunoperoxidase staining using monoclonal antibody to CD30 antigen

ALCL are usually CD45-positive and express one or more pan-T-cell antigens (CD2, CD3, CD5, CD7). ALCL may express *ALK* protein (Fig. 22–25).

Treatment and Prognosis

For the purposes of prognostic assessment and therapeutic selection, the NHLs can be grouped into two broad categories: the indolent lymphomas and diffuse aggressive lymphomas (see Tables 22–4 through 6). The indolent lymphomas are so designated because the median survival without therapy for this group of lymphomas is relatively long (7 to 9 years). The diffuse aggressive lymphomas are rapidly fatal without treatment (median survival is 6 to 12 months).

Therapy for the indolent lymphomas needs improvement.^{49–52} Few patients present with localized disease (stages I and II) treatable with radiotherapy. Approximately 90% of these lymphomas are stage III or IV at presentation, and many eventually relapse after standard or aggressive multiagent chemotherapy. Asymptomatic patients with advanced stage indolent lymphomas may be managed with a “watch and wait” approach. When symptoms warrant, or the lymphoma progresses to a higher grade, therapy is instituted.

Before the development of modern chemotherapeutic modalities, the indolent lymphomas were considered “favorable” lymphomas and the diffuse aggressive lymphomas were considered “unfavorable.” The situation is now reversed. The indolent lymphomas are recognized as clinically slow growing but often incurable with current therapies. On the other hand, the diffuse aggressive lymphomas are rapidly fatal if untreated but are potentially curable with aggressive treatment strategies. Long-term survival depends on achieving complete remission. For diffuse aggressive lymphomas, standard therapy is multiagent chemotherapy (e.g., R-CHOP) with radiation therapy, and cure is feasible, though there is

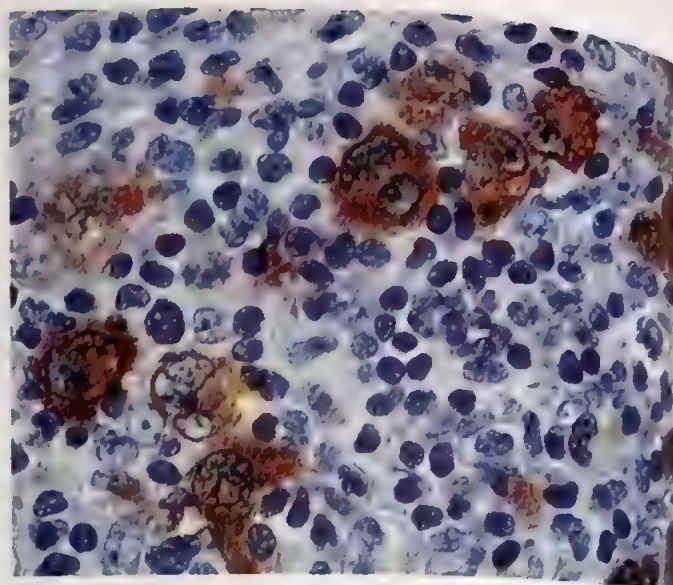


FIGURE 22-25 Expression of ALK-NPM protein in cytoplasm of neoplastic cells of ALCL (red-brown reaction product). Immunoperoxidase staining using monoclonal antibody to ALK-NPM protein (ALK-1).

also a risk of secondary (treatment-induced) myeloid leukemia that is usually resistant to therapy.

CRITICAL THINKING QUESTION

22-3 Would a diagnosis of non-Hodgkin lymphoma instead of Hodgkin lymphoma be desirable from a patient's perspective?

Acknowledgment

The author gratefully acknowledges the contributions of Dan M. Hyder, MD.

SUMMARY CHART

- The malignant lymphomas are a group of cancers that arise from cells of the lymphoid tissue (lymphocytes, histiocytes, reticulum cells).
- The Reed–Sternberg cell is the hallmark of Hodgkin lymphoma; the morphological features of the Reed–Sternberg cell include large size (up to 45 μ m in diameter), multinucleated, and inclusion-like nucleoli. The typical immunophenotype of Reed–Sternberg cells are CD30+, CD15+, and CD45–.
- An infectious agent found in some Hodgkin lymphomas is Epstein–Barr virus.
- Lymphocyte-predominant Hodgkin lymphoma consists of a mixture of small, normal-appearing lymphocytes; benign histiocytes; variant Reed–Sternberg cells called popcorn cells; or L&H cells.
- The most common type of Hodgkin lymphoma is nodular sclerosis, which is characterized by bands of collagenous fibrosis (sclerosis), classic Reed–Sternberg cells, and lacunar cells.
- Mixed cellularity Hodgkin lymphoma is characterized by a heterogeneous mixture of cells including lymphocytes, histiocytes, plasma cells, eosinophils, and Reed–Sternberg cells.
- Lymphocyte-rich Hodgkin lymphoma is characterized by Reed–Sternberg cells in a background of lymphocytes.
- Most patients with Hodgkin lymphoma present with nonpainful lymph node swelling.
- The World Health Organization (WHO) scheme relies on clinical, morphological, immunophenotypic, and genotypic features to classify the lymphomas.
- Follicular lymphoma (a form of non-Hodgkin lymphoma) has a mixture of lymphocytes, mainly centrocytes (small cleaved) and centroblast (large noncleaved) cells that have a B-cell immunophenotype (CD19+, CD20+, CD5–) and t(14;18) *IGH-BCL2*.
- Mantle cell lymphoma is characterized by small- to medium-sized lymphocytes with scant cytoplasm.

- irregular nucleus, and inconspicuous nucleoli; the cells express surface immunoglobulin, CD5, and CD43; the characteristic translocation is $t(11;14)(IGH-CCND1)$.
- The mucosa-associated lymphoid tissue (MALT) lymphomas are comprised of neoplastic marginal zone cells located in extranodal sites (stomach, salivary gland, lung, thyroid, and orbit) at and just under epithelial surfaces.
- The immunophenotype of Burkitt lymphoma is surface IgM+, CD10+, CD5-, CD23-, Bcl-2-, and TdT-.
- Characteristics of the systemic form of anaplastic large cell lymphoma include overexpression of ALK

protein, $t(2;5)(NPM1-ALK)$, CD30+, and infiltration of the lymph node sinuses by large atypical malignant cells.

- Primary cutaneous T-cell lymphoma is associated with Mycosis fungoides and Sezary syndrome.
- Diagnosis of lymphomas requires a variety of immunophenotypic tests, PCR, FISH, karyotyping, and light microscopy to differentiate between malignant lymphomas, B cell versus T cell, Hodgkin and non-Hodgkin lymphomas, and indolent versus aggressive forms.

CASE STUDY 22-1

A 68-year-old male auto mechanic presents to his primary care physician with a chief complaint of bilateral painless masses in the neck region of 6-month duration. He has a 120 pack-year smoking history, and his family history includes a father and brother with thyroid cancer. His past medical history is positive for rheumatoid arthritis. On physical examination, the patient is found to have bilateral supraclavicular and cervical lymphadenopathy consisting of matted groups of lymph nodes. The patient was afebrile and reported unintentional weight loss from 195 pounds to 180 pounds over a 6-month interval.

QUESTIONS

- Which of this patient's presentations and health history findings should raise flags for potential lymphoma?
- What diagnostic tests should be performed?
All parameters of the patient's complete blood count (CBC) and peripheral smear were normal. A chest radiograph demonstrated hyperinflation without parenchymal lung lesions. Fine-needle biopsy of a group of cervical nodes was performed. On microscopic examination there was diffuse effacement of lymph node architecture by a population of relatively uniform 20 to 40 μ m cells with round to slightly irregular nuclei and occasional prominent nucleoli. Immunophenotypically, the large cells expressed CD45, CD19, CD20, and a T-cell clone without T-cell antigens.
- What is the most likely diagnosis based on the available data?

- What additional studies should be performed before therapy is initiated?

Bilateral bone marrow biopsy specimens demonstrate several aggregates of abnormal cells similar to those observed in the lymph nodes, remaining distributed within the marrow. A radiographic scan of the abdomen and pelvis reveals para-aortic adenopathy and moderate enlargement of the spleen.

ANSWERS

- The patient's age and exposure to smoking for prolonged duration, combined with their familial history of thyroid issues and his autoimmune condition (rheumatoid arthritis), should lead the physician to order diagnostic tests for lymphoma.
- The formed elements (white blood cells, red blood cells, and platelets) of the peripheral blood often exhibit changes that are helpful in assessing the cause of lymphadenopathy; therefore, a CBC with peripheral smear evaluation is indicated. A chest radiograph helps search for intrathoracic disease, particularly lung lesions. The definitive test to distinguish benign from malignant processes is microscopic examination of tissue from the mass. This may be achieved by fine-needle aspiration or excisional biopsy; the latter preferred if lymphoma is high on the differential diagnostic list.
- Malignant lymphoma, diffuse large B cell (by WHO classification).
- For staging purposes, bilateral bone marrow biopsies and CT scanning of the abdomen and pelvis were performed.

REVIEW QUESTIONS

- Lymphoma is different from leukemia in that it originates in the:
 - Bone marrow
 - Breast tissue
 - Lymphatic tissue
 - Circulating lymphocytes
- What infectious agent is most commonly associated with the pathogenesis of Hodgkin's lymphoma?
 - Echovirus
 - Herpes simplex virus
 - Helicobacter pylori*
 - Epstein-Barr virus
- Which of the following is the cell characteristic of all types of classic Hodgkin lymphoma?
 - Lacunar cell
 - Popcorn cell
 - Reed-Sternberg cell
 - Sézary cell
- Which describes the Reed-Sternberg cell?
 - Small cell size
 - No nucleoli
 - Polylobated nucleus
 - Very little cytoplasm
- Popcorn cells are seen in which Hodgkin lymphoma?
 - Classic Hodgkin lymphoma
 - Nodular lymphocyte-predominant Hodgkin lymphoma
 - Nodular sclerosis classic Hodgkin lymphoma
 - Mixed cellularity classic Hodgkin lymphoma
- Lacunar cells are seen in which Hodgkin lymphoma?
 - Classic Hodgkin lymphoma
 - Nodular lymphocyte-predominant Hodgkin lymphoma
 - Nodular sclerosis classic Hodgkin lymphoma
 - Mixed cellularity classic Hodgkin lymphoma
- Which Hodgkin lymphoma has a female predominance?
 - Nodular sclerosis classic Hodgkin lymphoma
 - Mixed cellularity classic Hodgkin lymphoma
 - Nodular lymphocyte-predominant Hodgkin lymphoma
 - Classic Hodgkin lymphoma
- Fibrous bands are seen by microscopy in a mediastinal mass affecting an asymptomatic young woman most likely to have which type of Hodgkin lymphoma?
 - Nodular sclerosis
 - Lymphocyte-predominant
 - Mixed cellularity
 - Lymphocyte depletion
- Which type of chromosomal abnormality is seen in Burkitt lymphoma?
 - Deletion
 - Mutation
 - Translocation
 - Amplification
- Which is the most commonly diagnosed category of non-Hodgkin lymphoma?
 - Diffuse large B-cell lymphoma
 - Hairy cell leukemia
 - Lymphoplasmacytic lymphoma
 - Burkitt lymphoma
- A starry sky pattern of tingible-body macrophages is found in tumor tissue of which non-Hodgkin lymphoma?
 - Diffuse large B-cell lymphoma
 - Hairy cell leukemia
 - Lymphoplasmacytic lymphoma
 - Burkitt lymphoma
- Which of the following characterizes small lymphocytic lymphoma?
 - Aggressive but still generally curable
 - Usually CD25-, CD5+, CD23+, CD10-
 - Concomitant chronic lymphocytic leukemia
 - t(14;18)
- Mycosis fungoides is commonly associated with which T-cell lymphoma?
 - T-lymphoblastic lymphoma
 - Peripheral T-cell lymphoma
 - Primary cutaneous T-cell lymphoma
 - Anaplastic large cell lymphoma
- Translocations involving the *CCND1* gene is commonly associated with which B-cell lymphoma?
 - Small lymphocytic
 - Lymphoplasmacytic
 - Mantle cell
 - Marginal zone
- MALT lymphomas are most likely to have which of the following characteristics?
 - Extranodal involvement
 - T-cell immunophenotype
 - Blasts with fine nuclear chromatin
 - Reed-Sternberg cells
- A lymphoma expressing a monoclonal B-cell phenotype with coexpression of CD10 and translocation of the *BCL2* gene is most likely derived from which cells?
 - Follicular center cells
 - Mantle cells
 - Marginal zone cells
 - Interfollicular cells

REVIEW QUESTIONS—cont'd

17. Lymphoblastic lymphomas are most associated with which of the following?
 - a. Concomitant acute lymphoblastic leukemia
 - b. Differentiation to plasma cells
 - c. Clumped nuclear chromatin
 - d. Epstein-Barr virus genome in tumor cells
18. Reed-Sternberg cells of classic Hodgkin lymphoma usually express which of the following immunophenotypes?
 - a. CD45-, CD30+, CD15+
 - b. CD45-, CD30-, CD15-
 - c. CD45-, CD30-, CD15-
 - d. CD45-, CD30-, CD15-
19. Which of the following is a subtype of non-Hodgkin lymphoma?
 - a. Nodular sclerositis
 - b. Acute myeloid leukemia
 - c. Mantle cell
 - d. Infectious mononucleosis
20. Mycosis fungoides is commonly associated with which T-cell lymphoma?
 - a. T-lymphoblastic lymphoma
 - b. Peripheral T-cell lymphoma
 - c. Primary cutaneous T-cell lymphoma
 - d. Anaplastic large cell lymphoma
21. Which diagnostic analysis can aid most in determination of benign versus malignant lymphoma?
 - a. Light microscopy
 - b. Karyotyping
 - c. Immunophenotyping
 - d. Peripheral smear evaluation
22. The diffuse aggressive lymphomas are:
 - a. Incurable
 - b. Indolent
 - c. Treated with surgical resection
 - d. Responsive to chemotherapy
23. Which describes an indolent prognosis of non-Hodgkin lymphoma?
 - a. Median survival of 6 to 12 months
 - b. Clinically slow growing and very curable
 - c. Clinically slow growing, but often incurable
 - d. Clear therapies exist

See answers at the back of this book.

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Multiple Myeloma and Related Plasma Cell Disorders

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CHAPTER OUTLINE

Plasma Cell Development	Epidemiology	Nonsecretory Myeloma
Immunoglobulin	Etiology	POEMS Syndrome
Structure and Function	Pathophysiology	Waldenström Macroglobulinemia
Abnormal Monoclonal	Clinical Findings	Light-Chain Amyloidosis
Immunoglobulin Level Recognition	Diagnostic Criteria	Light-Chain Deposition and Heavy-
and Measurement	Laboratory Testing and Results	Chain Diseases
Laboratory Recognition and	Diagnostic Criteria	Summary Chart
Measurement	Staging	Case Study 23-1
Monoclonal Gammopathy of	Treatment	Case Study 23-2
Undetermined Significance	Variants of Plasma Cell Syndromes	Case Study 23-3
Smoldering Myeloma	Solitary Plasmacytoma	Review Questions
Multiple Myeloma	Plasma Cell Leukemia	References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

23-1 Explain how plasma cells contribute to immunity.

23-2 Describe the normal structure and function of immunoglobulin.

23-3 Correlate abnormal immunoglobulin production with clinical findings.

23-4 Identify common lab analyses utilized to assess immunoglobulin abnormalities.

23-5 Identify the clinical and lab findings in monoclonal gammopathies of undetermined significance.

23-6 Describe the clinical and lab findings required for classification of smoldering myeloma.

23-7 Describe the most common characteristics of a patient diagnosed with multiple myeloma.

23-8 Identify causes of pancytopenia in multiple myeloma.

23-9 Correlate lab findings with the clinical presentation of multiple myeloma.

23-10 Contrast lab findings and presentations of variants of plasma cell syndromes.

23-11 Compare Waldenström macroglobulinemia and plasma cell myeloma (multiple myeloma).

This chapter explains plasma cell development and immunoglobulin structure and function and briefly reviews selected plasma cell dyscrasias. The content focuses on pathophysiology and the clinical, laboratory, and radiological studies used to evaluate each disorder. The roles of cytogenetics, genetic testing, and molecular biology are also discussed. Staging and prognosis are introduced, but details of treatment are beyond the scope of this chapter.

Plasma Cell Development

Plasma cells are terminally differentiated B-lymphocytes that are responsible for the humoral immune response.¹ These cells

produce antibodies, called **immunoglobulins**, that respond to foreign pathogens with a high degree of specificity. A plasma cell can be distinguished from other mature B cells based on the presence of a dense, eccentric nucleus and ample cytoplasm containing large amounts of rough endoplasmic reticulum and Golgi apparatus.²

Hematopoietic stem cells commit in the bone marrow to the B-cell lineage, driven by specific transcription factors.³ DNA contains code for the components of the immunoglobulin molecule, including 40 variable (V) segments, 9 diversity (D) segments, 6 joining (J) segments, and a constant region. Light chains have a similar structure but do not have D segments.⁴

Rearrangement of immunoglobulin heavy chain genes enables pro-B cells to become pre-B cells. Light-chain clonal expansion and rearrangement occur next and allow cells to express surface immunoglobulin. Subsequently, immature B-lymphocytes leave the bone marrow, migrate to the peripheral blood, and rest in the G_0 (dormancy) phase of the cell cycle. On exposure to antigens that occur in the lymph nodes, the cells differentiate into short-lived “low affinity” plasma cells. A few become long-lived “memory B cells” and reside in the primary follicle of the lymph nodes. Once rechallenged with the same antigen, the primary follicles are transformed into secondary follicles with germinal centers. The memory B cells differentiate into centroblasts after immunoglobulin isotype switching and somatic mutations in the variable region of the immunoglobulin, generating “high-affinity” antibodies. Antibodies result in neutralization (blocking a pathogen’s ability to enter a host cell), opsonization (antigen-antibody bond promoting phagocytosis), and activation of complement cascade.⁴

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These cells differentiate into plasmablasts, leaving the lymph node and entering the peripheral blood, where the cells acquire several adhesion molecules such as CD56,

syndecan-1, collagen, ICAM-1, and leukocyte function-associated antigen 1, as well as vascular cellular adhesion molecule or fibronectin. These terminally differentiated plasma cells “home” back to the bone marrow and adhere to the stromal cells. These bone marrow plasma cells produce most of the serum immunoglobulin and have a life span approximately 1 month (Fig. 23-1).⁵

Immunoglobulin

Structure and Function

An intact immunoglobulin is composed of two identical heavy chains (50 kD) and two identical light chains (25 kD) (Fig. 23-2). These chains are held together by varying numbers of disulfide bonds. There are two light chain types—either kappa (κ) or lambda (λ); only one is found in any isotype.⁴ There are five types of heavy chains: gamma (γ), alpha (α), mu (μ), delta (δ), and epsilon (ϵ), and these heavy chains are designated as IgG, IgA, IgM, IgD, and IgE, respectively. The heavy polypeptide chains are further subdivided; IgG has four subclasses: IgG1, IgG2, IgG3, and IgG4, and IgA has two: IgA1 and IgA2. IgA and IgG aggregate in pairs, or *dimers*. IgM is a large molecule composed of five such units; it does not cross the placenta.

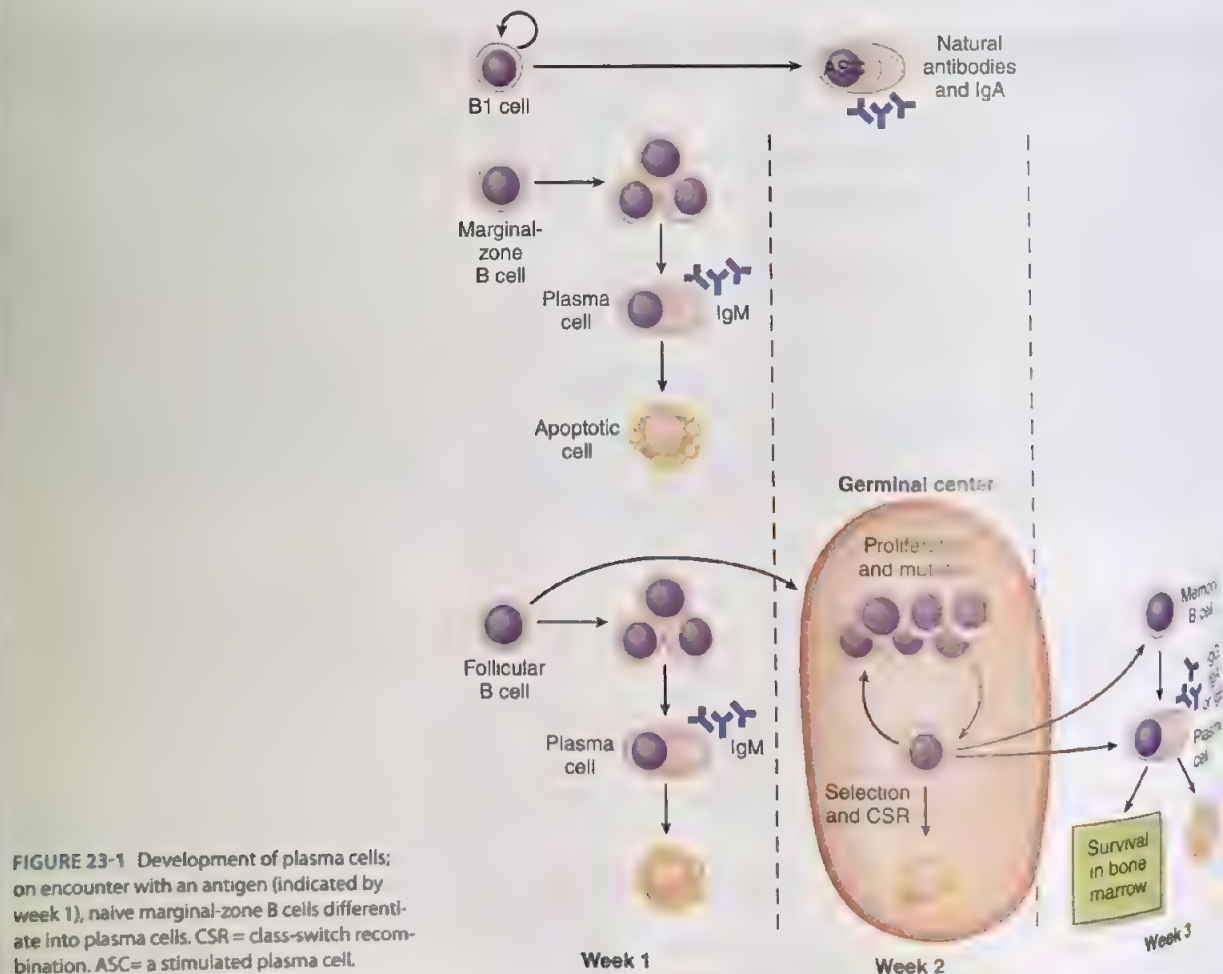


FIGURE 23-1 Development of plasma cells; on encounter with an antigen (indicated by week 1), naive marginal-zone B cells differentiate into plasma cells. CSR = class-switch recombination. ASC = a stimulated plasma cell.

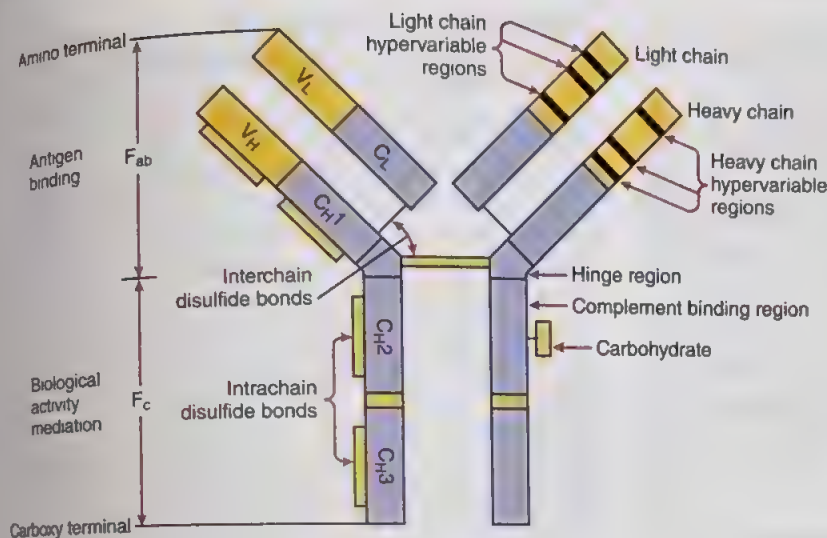


FIGURE 23-2 Structure of the basic immunoglobulin unit. V_H = variable region heavy chain; V_L = variable region light chain; C_H = constant region heavy chain; C_L = constant region light chain.

Each antibody has a specific role in the immune system. IgM is the initial “transient” antibody produced in response to infection; it becomes unmeasurable within weeks after it first appears. Later, IgG levels increase and are maintained in variable amounts after the antigenic stimulus is removed. High levels can be readily available on reexposure through memory cells. IgA is an antibody that provides protection of epithelial surfaces in the gastrointestinal tract and the airways. IgE antibodies are involved in allergic/hypersensitivity reactions. The role of IgD remains unclear, although emerging data suggest its role in regulation of immunological tolerance, protective B-cell response, and regulation of homeostasis at mucosal sites.⁷

Plasma cell neoplasms are characterized by clonal proliferation of a plasma cell population, leading to overproduction of a specific immunoglobulin. As a result, the normal ratio of κ to λ free light chains is altered. Excess involved free light chains, known as Bence Jones proteins, can be detected in the urine.

CRITICAL THINKING QUESTIONS

23-1 How can analysis of immunoglobulins be utilized to assess between initial infection and reinfection of a virus or foreign body?

See answers to all Critical Thinking Questions at the back of this book.

Abnormal Monoclonal Immunoglobulin Level Recognition and Measurement

Increased Production of Immunoglobulins: Hyperviscosity Syndrome

The presence of excess immunoglobulin can increase serum viscosity, resulting in higher resistance to blood flow, increased cardiac demands, and inadequate perfusion of end organs. Consequently, patients may experience confusion, headache, dementia, stroke, or coma. Failure of the heart to compensate for widespread hypoxemia, increased intravascular volume, and high pressure may lead to congestive heart

failure. Although the correlation between serum viscosity and clinical manifestations is not precise, clinical manifestations are rarely attributable to hyperviscosity if serum viscosity is less than 4 centipoises (CP) (normal value ≤ 1.8 CP). Most patients are symptomatic when serum viscosity is greater than 6 CP. Hyperviscosity is most commonly associated with Waldenström macroglobulinemia (WM), a clonal disorder that produces IgM protein, given the higher molecular weight (925 kDa) and pentad structure of IgM protein.

Therapeutic plasmapheresis is a technique that separates plasma from whole blood and can remove the excess immunoglobulins in symptomatic hyperviscosity.⁸

Hypogammaglobulinemia

Patients with plasma cell disorders can have decreased levels of normal “uninvolved” immunoglobulins, a condition known as **hypogammaglobulinemia**. One study demonstrated that 90% of patients with plasma cell myeloma had a reduction in at least one uninvolved immunoglobulin.⁹ Hypogammaglobulinemia leads to impaired immunity and increased susceptibility to infections, especially due to encapsulated organisms such as *Haemophilus influenza* and *Streptococcus pneumoniae*. Intravenous immunoglobulin (IVIG) can decrease the frequency of infectious complications in selected populations.

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Cryoglobulinemia

Cryoglobulins are serum immunoglobulins that precipitate reversibly on exposure to cold ($<37^\circ\text{C}$). Clinical manifestations of cryoglobulinemia include pain on cold exposure, purpura, arthralgias, and peripheral neuropathy. Serious complications including glomerulonephritis, hyperviscosity syndrome, and involvement of end organs (heart, gastrointestinal tract, nervous system) can occur. Cryoglobulinemia is mechanistically associated with monoclonal

polyclonal immunoglobulins, and can be classified into several subtypes:¹⁰

- Type I: Isolated monoclonal immunoglobulin (most commonly IgG or IgM). Associated with the following monoclonal gammopathies: monoclonal gammopathies of undetermined significance (MGUS), multiple myeloma, WM, CLL, and rarely non-Hodgkin's lymphoma.
- Type II: A combination of a polyclonal immunoglobulin and a monoclonal immunoglobulin (most commonly IgM) with rheumatoid factor activity. Associated with hepatitis C and human immunodeficiency virus (HIV).
- Type III: A combination of polyclonal immunoglobulins. Associated with connective tissue diseases.

Laboratory Recognition and Measurement Electrophoresis

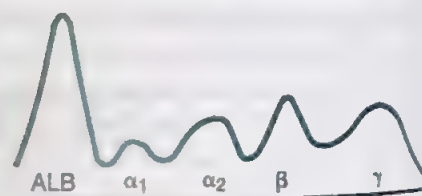
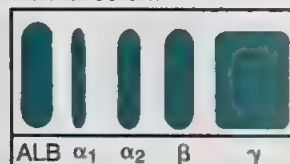
Albumin is the most abundant serum protein and is essential for transport;¹¹ however, immunoglobulins and other

serum proteins also occur in smaller quantities. Total serum protein is measured on routine automated chemistry panels. The measurement of albumin allows estimation of the immunoglobulin-containing fraction by subtraction (i.e., total protein – albumin = immunoglobulin fraction). A large difference in the value of total protein and albumin suggests that another protein is present in substantial quantity. It is important to differentiate between a polyclonal and monoclonal increase in immunoglobulin levels that is seen in infectious and plasma cell disorders, respectively. This is performed by a serum protein electrophoresis (SPEP), whereby serum is applied on particular medium and a charge is applied.¹² Proteins separate, based upon their electrical charge and size, into albumin, α , β , and γ globulins (Fig. 23–3). If a monoclonal immunoglobulin is present, a monoclonal peak in the gamma region, termed “M-spike,” will be present. SPEP allows for the detection and quantification of an M-spike. Similarly, a urine protein electrophoresis (UPEP) can be performed. Immunofixation (IFE) should always be performed in conjunction with the SPEP to further characterize a monoclonal protein.

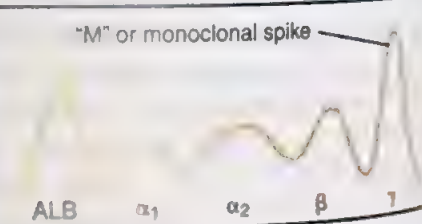
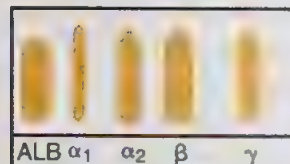
CELLULOSE ACETATE PATTERN

DENSITOMETER TRACING

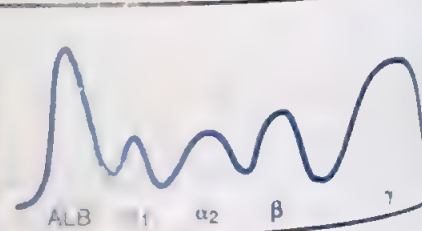
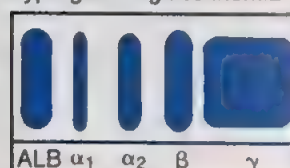
Normal serum



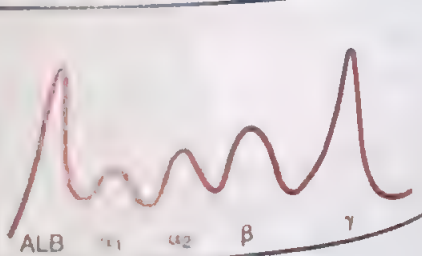
IgG Myeloma with γ spike and reduced albumin



Polyclonal hypergammaglobulinemia



Waldenström's macroglobulinemia with IgM spike



Hypogammaglobulinemia

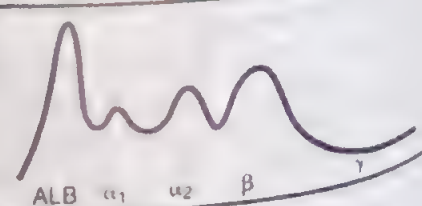
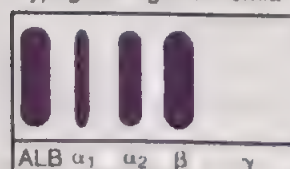


FIGURE 23-3 Patterns of serum protein electrophoresis showing characteristic patterns of normal serum, IgG myeloma with a γ -spike, polyclonal hypergammaglobulinemia, Waldenström's macroglobulinemia with IgM-spike, and the absence of antibody production seen in hypogammaglobulinemia.

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Immunofixation

Immunofixation (IFE) is used when investigating an abnormal M-spike on serum protein electrophoresis to identify the heavy chain and light chain involved with a high degree of sensitivity. After separation of the various proteins by electrophoresis into at least five separate lanes, monospecific antibodies, usually three for the heavy chain component (γ , α , μ) and two for the light chain component (κ and λ), are added. Precipitation of proteins (i.e., the antigen-antibody complex) is allowed to occur, followed by washing (non-precipitated proteins wash out) and staining of the remaining immunoprecipitated. An M-protein is characterized on IFE by the combined presence of a sharp, well-defined band associated with a single heavy-chain class and a sharp and well-defined band with similar mobility characteristics that reacts with either kappa or lambda light-chain antisera (Fig. 23-3).

Quantitative Immunoglobulins

Immunoglobulin classes are quantitated by rate nephelometry, which is based on measuring the degree of turbidity by light scatter caused by antigen-antibody complexes by mixing a patient's serum sample with an appropriate specific antibody reagent. Estimation of quantitative immunoglobulin by nephelometry does not allow for an assessment of monoclonality. Increased levels can be due to polyclonal or monoclonal elevations; clonality needs to be established using electrophoresis and immunofixation, as discussed in the preceding text. Quantitation of immunoglobulin using nephelometry is a useful adjunct to electrophoresis in patients with plasma cell dyscrasia.

Free Serum Light Chains

Measured free serum light chain (FLC) levels are affected by immunoglobulin production and clearance by the kidneys.¹³ While several conditions can influence the absolute concentration of κ and λ free light chains (immunosuppression, infection, inflammation, impaired renal clearance), only in monoclonal gammopathies is the κ/λ ratio altered. A κ/λ ratio of 0.26 to 1.65 was previously validated as the expected normal reference interval, although emerging data suggest a broader range may be acceptable in chronic kidney disease.¹⁴

Recently, a highly sensitive, automated immunoassay for measurement of FLC concentrations in serum has been used for identification and monitoring of patients with monoclonal gammopathy (Fig. 23-4). Serum FLC assays are sensitive in detecting abnormalities that otherwise may not be identified by routine electrophoresis and immunofixation. These tests are used for diagnosis and monitoring of light-chain-only myeloma, nonsecretory myeloma, and light-chain (AL) amyloidosis.¹⁵ FLC values are now utilized in risk stratification of monoclonal gammopathy of

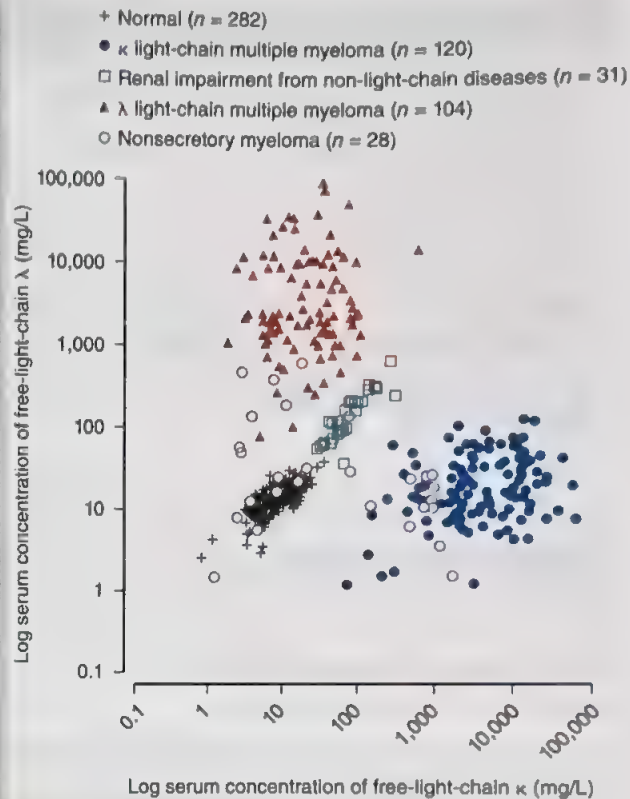


FIGURE 23-4 Concentrations of κ and λ free light chains in sera from healthy individuals and those with myeloma. (From Bradwell, AR: Serum test for assessment of patients with Bence-Jones myeloma. *Lancet*. 2003;361:489. Reprinted with permission from Elsevier.)

unknown significance (MGUS) and smoldering myeloma (SM).¹⁶

Bence-Jones Proteinuria

Imbalanced immunoglobulin production most frequently yields an excess of free light chains (κ/λ ratio). These light chains are rapidly metabolized in the blood, with increased production of FLC filtered into the urine as Bence-Jones proteinuria. The Bence-Jones protein is named after the physician who first noted its unique properties to precipitate at 40°C to 60°C, dissolve at 100°C, and precipitate again on recooling.¹⁷ Today, 24-hour urine collection is necessary to determine the total amount of protein excreted in the urine per day and to quantitate the Bence-Jones proteins. Excess serum FLC can deposit in the kidneys and lead to proteinuria and kidney failure.

CRITICAL THINKING QUESTION

23-2 Why does an M-spike on serum protein electrophoresis need further analysis?

BOX 23-1 Classification of Monoclonal Gammopathies**Monoclonal Gammopathies of Undetermined Significance
Malignant Monoclonal Gammopathies**

- Multiple myeloma (IgG, IgA, IgD, IgE, and free light chain)
- Smoldering myeloma
- Plasma cell leukemia
- Nonsecretory myeloma
- Osteosclerotic myeloma (POEMS syndrome)

Plasmacytoma

- Solitary plasmacytoma of bone/extramedullary plasmacytoma

**Waldenström Macroglobulinemia
Heavy-Chain Diseases**

- IgA (α -HCD)
- IgG (γ -HCD)
- IgM (μ -HCD)

Primary Light-Chain Amyloidosis**Monoclonal Gammopathy of Undetermined Significance**

Box 23-1 outlines the classification of monoclonal gammopathies: MGUS, malignant monoclonal gammopathies, plasmacytoma, Waldenström macroglobulinemia, heavy-chain diseases, and primary light-chain amyloidosis.

Monoclonal gammopathy of unknown significance (MGUS) is a premalignant clonal disorder with a 1% annual risk of malignant transformation.¹⁸ Non-IgM MGUS and IgM MGUS transform typically into multiple myeloma and Waldenström macroglobulinemia, respectively.

The incidence of MGUS varies by age, sex, and race. MGUS is diagnosed in approximately 1% of individuals older than the age of 50 and up to 3% of those older than the age of 70. MGUS is defined by the presence of a serum M-protein <3 g/dL, urine M-protein <500 mg/24h, $<10\%$ plasma cells on bone marrow biopsy, and absence of end-organ damage secondary to myeloma. A new term, **monoclonal gammopathy of renal significance (MGRS)**, was coined in 2012 to describe a monoclonal gammopathy where the secreted immunoglobulin deposits in the kidney without overt myeloma.¹⁹

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Once MGUS has been identified on initial screening tests (SPEP, SIFE, immunoglobulins, free light chains), risk scores can be calculated to determine appropriate monitoring strategy (Table 23-1).²⁰ High-risk patients, such as the elderly and individuals with comorbidities, extramedullary disease, and plasma cell leukemia (PCL), require skeletal imaging and bone marrow biopsy to evaluate for malignant transformation.

TABLE 23-1 Determining Risk in Monoclonal Gammopathy of Unknown Significance Risk Stratification

Risk Group	Absolute Risk of Progression to Multiple Myeloma in 20 Years
Low risk 1) Serum M-protein <1.5 g/dL 2) IgG subtype 3) Serum free light-chain ratio, 0.26-1.65	5%
Low-intermediate risk (1 risk factor)	21%
High-intermediate risk (2 risk factors)	37%
High risk (3 risk factors)	58%

Source: Adapted from Rajkumar SV, Kyle RA, Therneau TM, Melton LJ, 3rd, Bradwell AR, Clark RJ, et al. Serum free light chain ratio is an independent risk factor for progression in monoclonal gammopathy of undetermined significance. *Blood*. 2005;106(3):812-7.

Smoldering Myeloma

In the spectrum of plasma cell neoplasms, **smoldering myeloma (SM)** is the intermediate stage between MGUS and multiple myeloma. For diagnosis, patients must demonstrate high burden of monoclonal protein (M-protein ≥ 3 g/L, urinary monoclonal protein ≥ 500 mg per 24 hour) and/or 10% to 60% clonal plasma cells in the bone marrow. Importantly, patients must not have any evidence of end-organ damage (see Box 23-2). Patients need careful follow-up as the risk of progression to multiple myeloma is approximately 10% per year for the first 5 years, then decreasing risk of 5% per year for the subsequent 5 years, and 1% per year thereafter. There is much debate regarding whether SM should receive myeloma-directed therapy, and multiple clinical trials are ongoing to address this question. Currently, there is insufficient evidence to suggest routine treatment for patients with SM.

Multiple Myeloma

Multiple myeloma is the most common plasma cell dyscrasia, affecting terminally differentiated B cells. The biology of multiple myeloma suggests a multistep process as illustrated by the clinical progression from MGUS to symptomatic multiple myeloma (Fig. 23-5). The initial mutation event is thought to occur in the germinal center during isotype class switching and somatic hypermutation.²¹ Plasma cells are generally localized in the bone marrow until late in the disease when the cells grow independent of the bone marrow microenvironment, usually causing a more aggressive clinical presentation. Multiple myeloma is associated with other complications including immunosuppression, infection, pathological fracture, and cord compression. With refinement in treatment (novel therapies, triplet regimens, and autologous hematopoietic cell

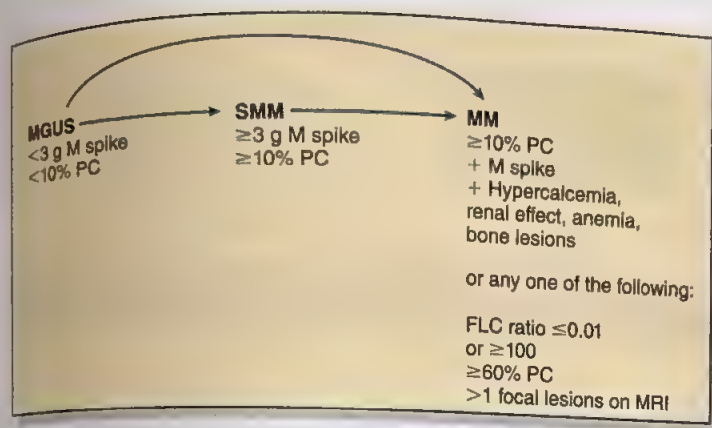


FIGURE 23-5 Progression from monoclonal gammopathy of undetermined significance (MGUS) to smoldering myeloma (SMM) to malignant myeloma (MM). PC = plasma cells; M spike = monoclonal spike; FLC = free light chains; MRI = magnetic resonance imaging.

transplant), the median overall survival in multiple myeloma now exceeds 5 years.²²

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Epidemiology

Multiple myeloma accounts for 10% of all hematological malignancies and 1% of all cancers, with an estimated 24,280 to 30,330 new cases and 12,650 deaths in 2016.²³ The median age at diagnosis is between 66 and 70 years, with the disorder extremely rare (<0.02%–0.03%) in individuals under 30 years of age. MGUS and multiple myeloma both are twice as frequent in African Americans compared with European Americans but with similar rates of malignant transformation.

Etiology

The underlying etiology of multiple myeloma remains unknown. Many medical conditions associated with chronic stimulation of the immune system, such as repeated infections, allergic conditions, or autoimmune disease, have been reported to increase the risk of multiple myeloma. Human herpes virus 8 (KSHV/HHV8) and hepatitis C have been considered in the pathogenesis of multiple myeloma. Biologically, it is plausible that the antigen-driven response to the viral infection and the associated increased levels of cytokines (e.g., interleukin [IL]-6) and angiogenic factors (e.g., vascular endothelial growth factor and basic fibroblastic growth factor) can initiate and sustain the malignant clone in multiple myeloma.²⁴

Prolonged low-dose exposures, as seen in factory workers and in those living near nuclear facilities, may increase the risk of multiple myeloma. Other occupations with a high risk of multiple myeloma include workers in the metal, rubber, wood, leather, paint, and the petroleum industries. The strongest occupational association for multiple myeloma, however, is agricultural work.²⁵ Plasma cell dyscrasias, including multiple myeloma and Waldenström macroglobulinemia, have been known to cluster in families, although neither specific genetic link nor environmental exposures have been clearly identified.²⁶

Pathophysiology

There are three primary processes that lead to end-organ damage in multiple myeloma. First is the expanding plasma cell mass in the bone marrow, second is the overproduction of monoclonal immunoglobulin, and third is the overproduction of various cytokines that affect bone structure and function (Fig. 23–6). In the following section, each process is discussed further.

Plasma Cell Expansion

As the plasma cell establishes a malignant clone, normal bone marrow is gradually replaced by the expanding malignant plasma cell colonies. As this process progresses, normal blood cells decrease in number, a condition referred to as pancytopenia. Typically, anemia (decrease in red blood cells) occurs first, followed by thrombocytopenia (decrease in platelets), and subsequently neutropenia. Anemia can result in fatigue, shortness of breath, and rapid heart rate. Thrombocytopenia results in delayed hemostasis, with resultant prolonged bleeding and easy bruising. Neutropenia increases susceptibility to infections.

The expanding plasma cell population in the bone marrow can result in destruction of cortical bone. Stretching of the overlying nerve-rich periosteum can be painful. Notably, pain is the most common presenting symptom in more than two-thirds of multiple myeloma patients. Tumor growth may extend beyond the boundaries of the bone to compress adjacent neurological structures, leading to nerve root impingement or spinal cord involvement. Clonal plasma cell proliferation may even occur outside the bone and in a soft tissue site, a condition known as **extramedullary plasmacytoma**.

Bone Marrow Stroma

The bone marrow microenvironment plays an important role in supporting the malignant plasma cells in multiple myeloma by promoting their growth and preventing apoptosis (Fig. 23–7). Adhesion of myeloma cells to the stromal cells induces secretion of several **cytokines** (proteins that promote or inhibit cell functions) from the stromal cells, endothelial cells, and/or osteoclasts as well as from the plasma cells themselves. These molecules play an important role in multiple myeloma pathogenesis and mediate many of the disease signs such as bone destruction, tumor cell proliferation, drug resistance, and genetic inst

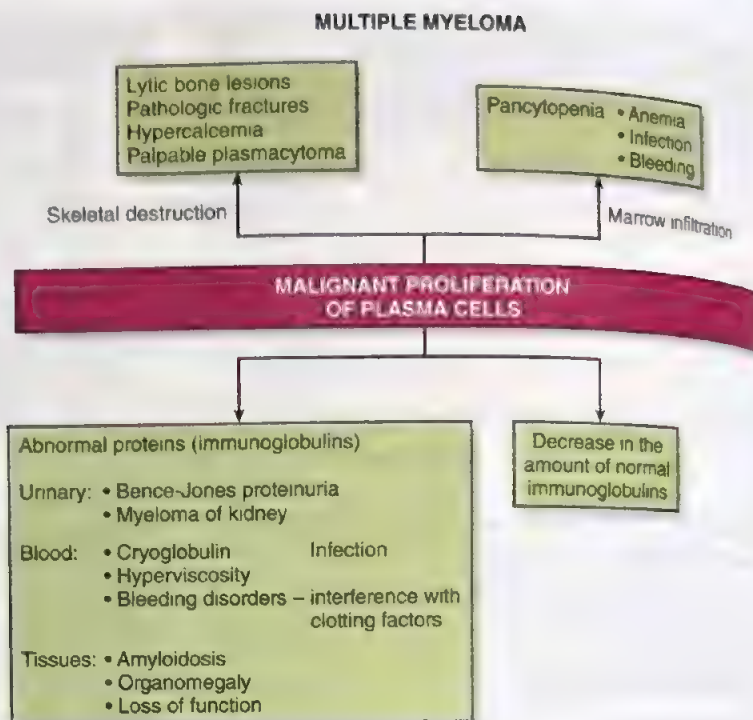


FIGURE 23-6 Mechanisms of disease in multiple myeloma. Skeletal destruction, abnormal immunoglobulin production, marrow failure, and decreased production of normal immunoglobulin all play a role.

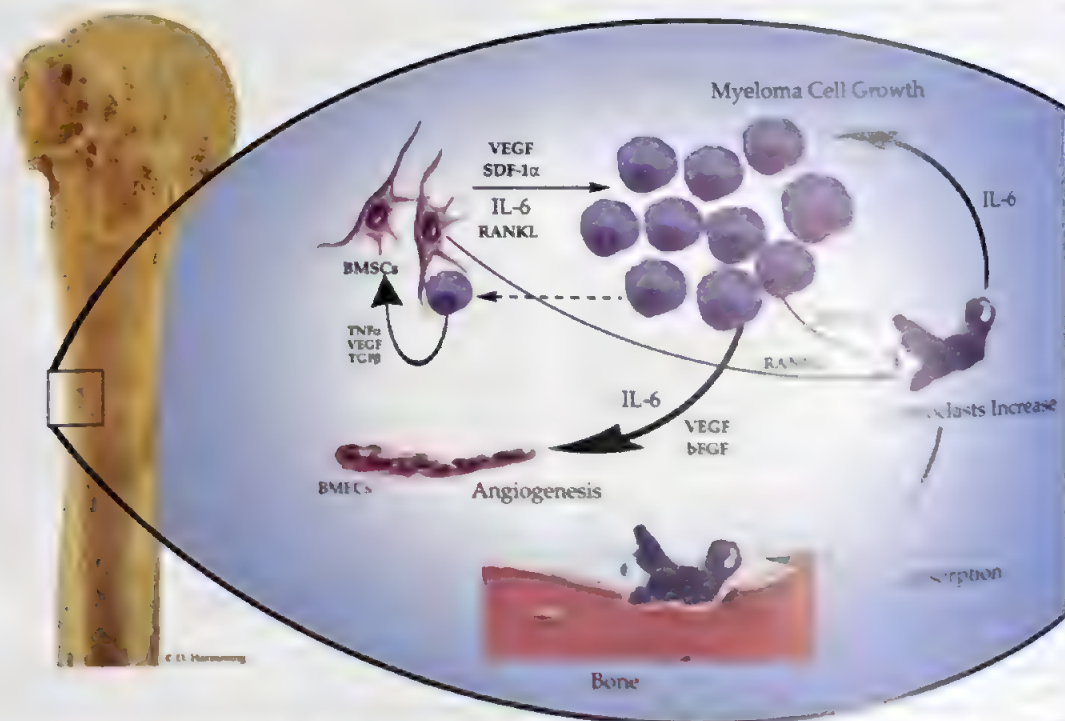


FIGURE 23-7 Bone marrow microenvironment. Effects of cytokines and transforming growth factors on the bone marrow (BM) microenvironment in patients with multiple myeloma (MM). Bone marrow stromal cells (BMSC) secrete IL-6, VEGF, SDF-1α, and RANKL. This IL-6 secreted in the BM microenvironment promotes the binding of MM cells to the BMSCs augmenting more secretion of IL-6 and other cytokines from the BMSCs and MM cells, for example, TNFα, TNFα, and VEGF from malignant myeloma cells enhance IL-6 secretion from BMSCs. The MM cells secrete IL-6, VEGF, bFGF, TNFα, and MIP-1α. The IL-6, VEGF, and bFGF secreted by the MM cells and the VEGF from BMSCs initiate angiogenesis in the marrow endothelial cells. The cytokines MIP-1α secreted by the MM cells and RANKL secreted by BMSCs initiate or activate osteoclast formation leading to bone resorption and bone destruction in MM patients. Osteoclasts also secrete IL-6, inducing the growth of the malignant clone of MM cells. IL-6, TNFα, and VEGF – vascular endothelial growth factor, SDF-1α – stromal cell-derived factor alpha, RANKL – receptor activator of TNFα, TNFα – tumor necrosis factor alpha, bFGF – basic fibroblast growth factor, TNFα – tumor necrosis factor alpha, TGFβ – transforming growth factor beta, MIP-1α – macrophage inflammatory protein-1α.

of plasma cells. The details of each cytokine and the various pathways involved are beyond the scope of this review. In brief, they include osteoclast activating factors, such as IL-1 β , IL-6, and the tumor necrosis factor (TNF) family proteins, as well as vascular endothelial growth factor, transforming growth factor- β , and insulin-like growth factors.²⁷

Bone Disease in Multiple Myeloma

Normal bone is a dynamic structure undergoing continuous renewal through the resorption of old bone by osteoclasts and the formation of new bone by osteoblasts. As a result, lytic lesion as well as osteoporosis can occur, the latter of which can be complicated by pathological fracture, vertebral height loss, and spinal cord compression. Multiple myeloma bone disease results from dissociation of bone absorption and formation secondary to stimulation of osteoclasts and inhibition of osteoblasts, respectively. Myeloma cells adhere and interact with bone marrow stromal cells resulting in the production of osteoclast-activating factors, including IL-1 β , IL-6, and IL-3 and tumor necrosis factor (TNF); these cytokines stimulate the TNF-related activation-induced cytokine (TRANCE).²⁸

osteoprotegerin. Osteoprotegerin is a cytokine and member of the TNF receptor superfamily. It is also known as osteoclastogenesis inhibitory factor (OCIF). The balance of TRANCE/osteoprotegerin is totally disrupted in multiple myeloma patients leading to overproduction of TRANCE and inactivation of osteoprotegerin by binding to syndecan-1 secreted by plasma cell surface (Fig. 23–8).²⁸ Recently, inhibition of osteoblasts was established as a major contributor to bone disease in myeloma. Dickkopf 1 (DKK1), an inhibitor of the Wnt signaling pathway, which is crucial for osteoblast differentiation, leads to reduced bone formation. In the Wnt signaling pathway, signaling molecules (Wnt proteins) regulate morphology, proliferation, motility, and cell fate.

Hypercalcemia

Calcium balance plays a critical role in regulation of cellular function. With increased bone turnover, calcium is released in the blood, causing several symptoms. Early symptoms include constipation and cramping from altered motility of the intestine, polyuria, dehydration, and muscle weakness. Increased calcium in the urine (hypercalciuria) can lead to formation of kidney stones and kidney failure. Altered mental

ADVANCED CONTENT

TRANCE is responsible for the activation and maturation of osteoclasts. TRANCE activity is blocked by

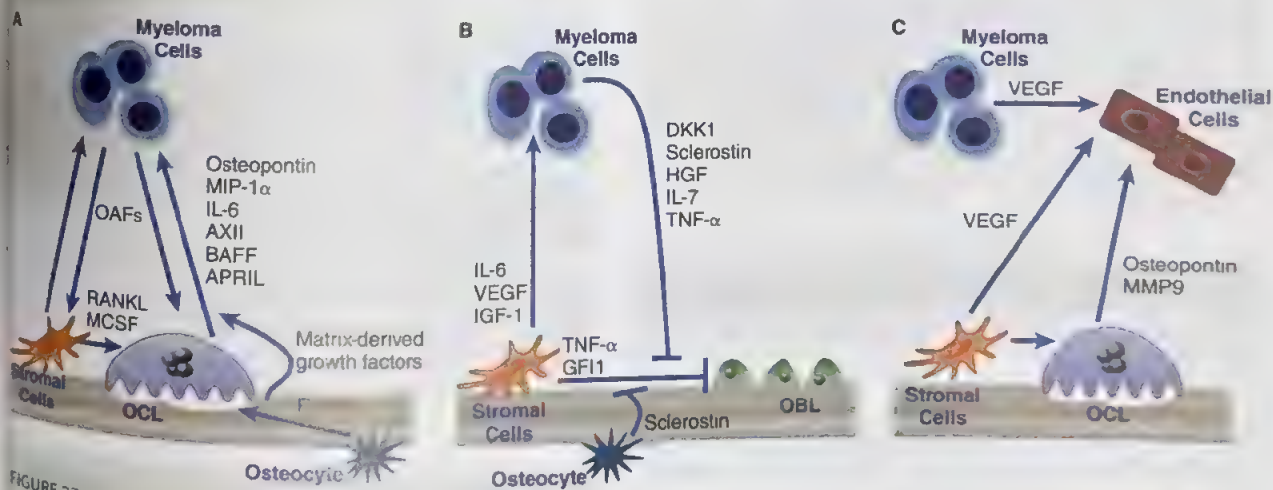


FIGURE 23-8 Cellular interactions in the bone marrow microenvironment in myeloma bone disease. The bone marrow microenvironment in myeloma includes osteoblasts (OBL), osteoclasts (OCL), stromal cells, endothelial cells, and osteocytes. Multiple interactions within the bone marrow microenvironment in myeloma are responsible for the abnormal bone remodeling of multiple myeloma bone disease (MMBD). (A) OCL Activation in MMBD. Myeloma cells directly stimulate OCL formation and induce cells in the marrow microenvironment to produce factors that drive OCL formation. Osteoclast-activating factors (OAFs) produced by myeloma cells include RANKL, MIP-1 α , IL-3, and TNF- α . Myeloma cells also induce marrow stromal cell production of growth factors that enhance OCL formation including RANKL, MCSF, and (not pictured) IL-6 and TNF- α and decrease production of OCL inhibitory factors, such as OPG. OCLs secrete soluble factors such as osteopontin, MIP-1 α , IL-6, AXII, BAFF, and APRIL that stimulate tumor growth. In addition, factors produced by marrow stromal cells and OCL promote tumor growth through direct action on myeloma cells. Osteocytes also regulate osteoclastogenesis and bone resorption through expression of RANKL. Finally, the bone destructive process releases bone matrix-derived growth factors such as TGF β , IGFs, FGF, PDGFs, and BMPs that increase the growth of myeloma cells, further exacerbating the osteolytic process. (B) OBL Suppression in MMBD. MM cell-derived OBL-inhibitory factors include DKK1, sclerostin, HGF, IL-7, and TNF- α . MM cells also induce other cells in the marrow microenvironment to increase production of OBL suppressors such as sclerostin (from osteocytes), and TNF- α and GFI1 (from marrow stromal cells). Myeloma cells also induce marrow stromal cells to produce factors that support the myeloma cells, including IL-6, VCAM1, VEGF, and IGF-1. (C) Angiogenesis is enhanced in MMBD. Angiogenesis is enhanced in MM. OCL and endothelial cells are closely apposed in the bone marrow microenvironment, and increased OCL activity appears to contribute to both the increased angiogenesis in MM as well as to tumor growth. Endothelial cell proliferation is enhanced by angiogenic factors such as VEGF produced by myeloma cells and stromal cells. Osteoclasts also secrete angiogenic factors, such as osteopontin and MMP9. (Adapted from Silbermann, Rebecca, and David Roodman. Myeloma bone disease: Pathophysiology and management. *Journal of Bone Oncology*. 2013;2(2):59-69. doi:10.1016/j.jbo.2013.04.001)

status can occur with significant hypercalcemia. The severity of the symptoms is dependent on both the level of calcium and rate of rise. Hydration, forced diuresis, and bisphosphonate therapy are among the therapies for hypercalcemia.

Clinical Findings

The signs and symptoms and possible causes of multiple myeloma are outlined in Table 23-2. Anemia can lead to weakness, loss of energy, cognitive slowing, and tachycardia in severe cases. One study demonstrates a seven-fold increase of infection in myeloma patients compared with matched controls.³⁰ Recurrent unexplained infections could warrant initial myeloma workup. Patients may have symptoms related to hypercalcemia, renal insufficiency, or amyloidosis. Bone pain is the most common presentation in multiple myeloma. Spinal cord compression from extramedullary plasmacytoma is an acute emergency that should be diagnosed and treated promptly to prevent long-term disability and paralysis. Immediate magnetic resonance imaging (MRI) or computed tomographic myelography of the entire spine must be done with appropriate follow-up treatment by radiotherapy or neurosurgery to avoid permanent damage.

The serum creatinine concentration is elevated in 20% of patients at diagnosis (>2 mg/dL).³¹ The most common cause of renal insufficiency in multiple myeloma is the precipitation of monoclonal light chains in the renal tubules called **cast nephropathy**. Other causes of renal dysfunction in multiple myeloma include deposition of light chains in the kidney parenchyma (light-chain deposition disease) and amyloid fibrils in AL amyloidosis. Acute kidney injury (AKI) can also occur due to various insults including but not limited to contrast dye, medication, and hypercalcemia.

TABLE 23-2 Signs and Symptoms of Multiple Myeloma

Signs and Symptoms	Possible Causes
Bone pain (68%)	Fracture, lytic lesions
Easy fatigue (62%)	Anemia (73%)
Polyuria (30%)	Hypercalcemia (13%)
Nausea and vomiting	Renal failure, hypercalcemia
Recurrent infections	Low normal Ig levels
Paraplegia	Cord compression
Confusion (15%)	Hyperviscosity, hypercalcemia
Bleeding	Thrombocytopenia
Arrhythmia (7%)	Amyloidosis (4%)
Peripheral neuropathy	Amyloidosis
Fever	Infection
Renal insufficiency (19%)	Cast nephropathy, light chain deposition

Source: Modified from Kyle, RA, et al. Review of 1027 patients with newly diagnosed multiple myeloma. *Mayo Clin Proc.* 2003;78:21.

Laboratory Testing and Results

Complete Blood Count and Peripheral Blood Smear

The automated complete blood count (CBC) is an easily available assessment of bone marrow function. The most common finding in multiple myeloma is a decrease in the number of red blood cells with no change in size (normocytic normochromic anemia). Normocytic normochromic anemia is covered in great detail in Chapter 6: Anemia. On peripheral smear examination, the most characteristic finding in multiple myeloma is **rouleaux** formation of the red blood cells (stacking of red blood cells together like coins) (Fig. 23-9) caused by increased amounts of immunoglobulin in the blood leading to red blood cells adhering to each other. The same phenomenon results in the increased erythrocyte sedimentation rate. Circulating plasma cells, if seen, are associated with a poor prognosis. Later in the disease, replacement of the bone marrow with plasma cells may also cause teardrop-shaped red blood cells by blood smear.

Chemistry Studies

Routine chemistry panels are essential in evaluating myeloma patients. Serum blood urea nitrogen (BUN) and creatinine measures the kidney function that is often affected in myeloma. Serum lactate dehydrogenase (LDH) is a nonspecific marker of tissue breakdown, and elevated levels are associated with shorter survival. Calcium levels are often elevated in myeloma patients, indicating bone destruction. Calcium is primarily bound to albumin; the unbound or free calcium is the major cause of symptoms. Calcium levels must always be interpreted with the albumin level in mind. For each mg/dL of albumin below normal, the serum calcium should be increased by 0.8 mg/dL. Albumin levels can be low in multiple myeloma due to inhibition by IL-6 as well as loss in the nephrotic kidney. In either case, albumin level is a major prognostic criterion in multiple myeloma.

β_2 -Microglobulin (β_2 M) is the light chain of the histocompatibility leukocyte antigen (HLA). It is the most useful predictor of tumor load and disease activity, thereby predicting the prognosis of multiple myeloma patients. C-reactive protein is another nonspecific marker of disease activity, mostly



FIGURE 23-9 Peripheral blood showing marked rouleaux formation, the "stacked-coin" appearance of the red blood cells.

reflecting the activity of IL-6, an important growth factor for myeloma cells. Its prognostic value is limited by the lack of specificity.

Electrophoresis and Immunofixation

As extensively discussed earlier, serum and urine protein electrophoresis, immunofixation, quantification of kappa and lambda light chains, and quantitative immunoglobulins are the standard parts of diagnostic workup of multiple myeloma. The incidence of nonsecretory myeloma in which there is no detectable monoclonal protein has decreased to <1% with the increased sensitivity of the current techniques. IgG is the immunoglobulin produced in more than 50% of cases, followed by IgA (20%); 20% produce only light chains and IgD is rarely seen in <1%. Two immunoglobulin classes can be detected in "biconal myeloma," which represents 3% of all myeloma cases and reflects the coexistence of two malignant clones.

Bone Marrow Examination

Bone marrow biopsy is essential in diagnosis of multiple myeloma. Biopsy findings in multiple myeloma include increased numbers of clonal plasma cells, often forming "sheets," with immature, binucleate, and large cells (Figs. 23-10 and 23-11). Certain features such as flame cells (large, intensely staining plasma cells) can suggest IgA subtype (Fig. 23-12). A differential count of marrow cells (usually 500 cells are counted) is performed to establish percentages of various cell types. Patients with myeloma have a variable level of plasmacytosis ranging from 10% to 100%. The bone marrow involvement could be patchy, thus the highest percentage from the aspirate or the core specimen is used for diagnosis.

Malignant plasma cells have a characteristic immunophenotype. In addition to staining positively for cytoplasmic immunoglobulin, these cells stain positive for CD38, CD56 (neural cell adhesion molecule), and CD138 on their surfaces. They are usually negative for surface immunoglobulin and the pan-B-cell antigen CD19; 15% to 20% will stain positively for CD20 and CD52. Special intracellular stains demonstrate clonality with either κ or λ restriction.

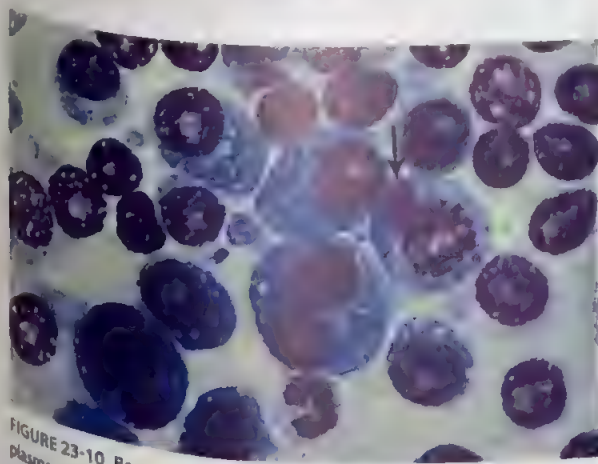


FIGURE 23-10 Bone marrow aspirate showing atypical and binucleated plasma cells and Russell bodies (arrow).

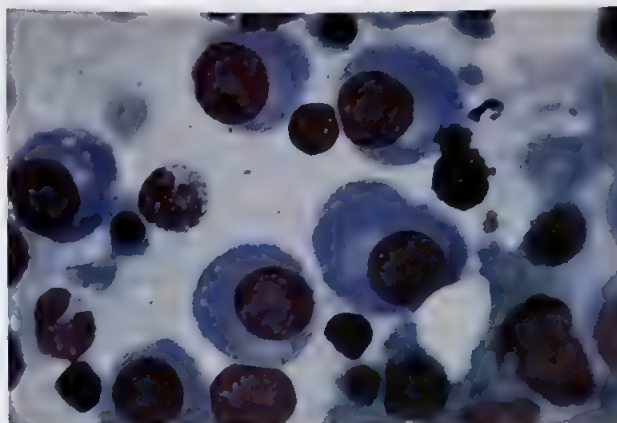


FIGURE 23-11 Bone marrow biopsy sample showing replacement of marrow by plasma cells.

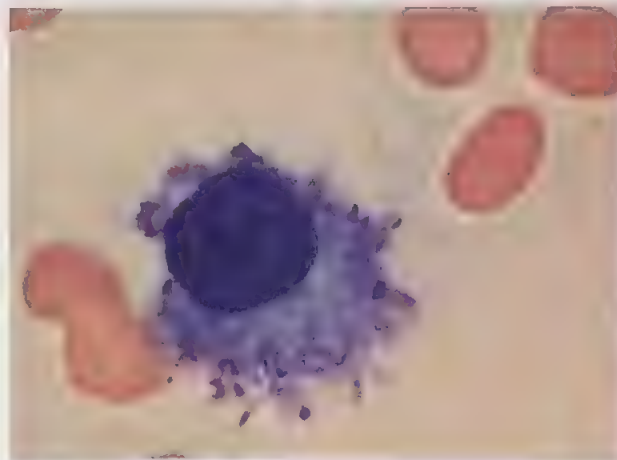


FIGURE 23-12 Flame cell, from a patient with IgA myeloma. Note: the red magenta hue to the periphery of the neoplastic plasma cell giving the cell this characteristic name. Image source: Girish Venkataraman, MD, MBBS. Used with permission of the American Society of Hematology (ASH).

CRITICAL THINKING QUESTION

23-3 Why is extreme bone pain the most common symptom upon presentation in most multiple myeloma patients?

Genomic Abnormalities

Chromosomal abnormalities are common in malignant plasma cells and are broadly divided into two main categories: hyperdiploidy and nonhyperdiploidy. Nonhyperdiploid plasma cells often harbor abnormalities involving 14q32, which is the locus of the immunoglobulin heavy chain (*IgH*) gene. Although coexistence of hyperdiploidy and translocations of *IgH* gene is rarely reported, they are considered mutually exclusive. Cytogenetic abnormalities are prognostic, thus interphase fluorescence in situ hybridization (FISH) studies are routinely incorporated into the initial diagnostic workup of multiple myeloma (Fig. 23-13).

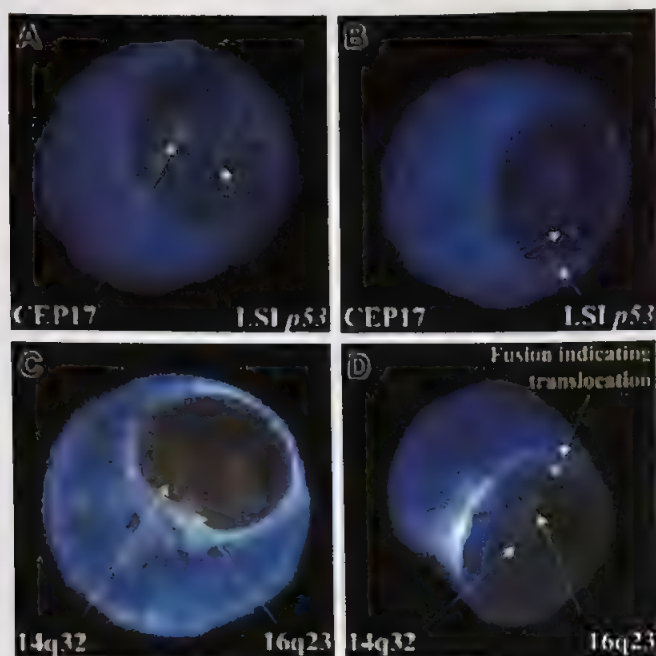


FIGURE 23-13 PCs with both the normal and abnormal pattern of hybridization. The depicted PCs show (A) a cell with the normal configuration of 2 pairs of signals for the probes localizing to the centromere 17 (CEP17; aqua) and the 17p13.1 (LSI p53) (red) probe. (B) A cell with deletion of 17p13.1. There are two green signals arising from the centromeric probe but only one red signal from the p53 locus probe. (C) A normal configuration of probes used to detect the t(14;16)(q32;q23). The locus-specific 14q32 probes are labeled in green, and the 16q23 probes are labeled in red. (D) A cell with fusion of probes for 14q32 (green) and 16q23 (red). (From Fonseca, R, et al. Clinical and biological implications of recurrent genomic aberrations in myeloma. *Blood* 2003;101:4570. Reprinted with permission from the American Society for Hematology.)

ADVANCED CONTENT

IgH translocation is the most frequent abnormality of multiple myeloma, which is reported in 45% of cases. This abnormality is likely an early key event in the development of malignant plasma cells, as it occurs at the time of isotype switching of the IgH region. The five most common translocations, in their order of prevalence, are t(11;14) (q13;q32), t(4;14)(p16.3;q32.3), t(14;16)(q32.3;q23), t(14;20)(q32;q11), and t(6;14)(p25;q32) (Table 23-3). The t(4;14), t(14;16), and t(14;20) translocations are associated with poor prognosis (Fig. 23-14).³¹ Notably, t(11;14)

TABLE 23-3 Common Chromosomal Translocations in Multiple Myeloma

Translocations	Affected Gene
t(11;14)	<i>Cyclin D1</i>
t(4;14)	<i>MGFR-3</i> and <i>MMSET</i>
t(6;14)	<i>Cyclin D3</i>
t(14;16)	<i>C-MAF</i>
t(14;20)	<i>MAF-B</i>

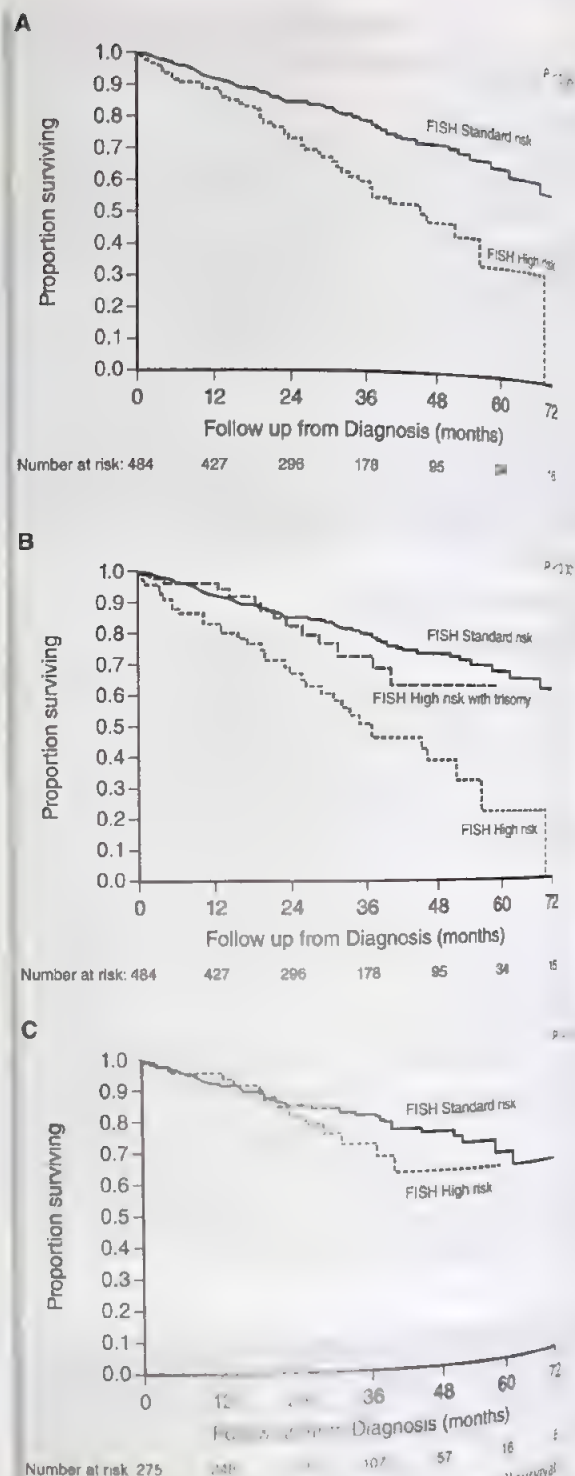


FIGURE 23-14 Kaplan-Meier curves demonstrating overall survival (OS) from diagnosis based on various risk factors. (A) Comparison of OS between patients with standard-risk MM (n = 370) based on FISH testing with those with high-risk MM (n = 114). (B) Comparison of OS between those with standard-risk MM (n = 370), high-risk MM with any trisomy (n = 66), high-risk MM without any concurrent trisomy (n = 48), and high-risk MM with any trisomy (n = 275) with or without high-risk FISH features. (Adapted from Kumar S, Fonseca R, et al. Trisomies in multiple myeloma: impact on survival in patients with high-risk cytogenetics. *Blood*. 2012;119(9):2100-5. doi: 10.1182/blood-2011-11-390658. Erratum 2012 Jan 10. Erratum in. *Blood*. 2014;123(10):1621. PMID: 22234061. PMID: PMC3311247.)

(q13;q32) involves the translocations of *IgH* and B-cell leukemia/lymphoma 2 (*BCL2*) genes, and is unique due to the potential treatment implications with a *BCL2* inhibitor, venetoclax.³²

Hyperdiploidy is also a common finding in multiple myeloma and prognostically considered standard risk. As previously described, *IgH* translocations and hyperdiploidy are generally mutually exclusive. In addition to *IgH* translocations and hyperdiploidy, monosomy 13, 17p deletion (loss of TP53), and gain of 1q are significant chromosomal findings that confer poor prognosis. These findings occur more commonly with *IgH* translocation but can also be seen with hyperdiploidy.

Wider availability of next generation sequencing (NGS) has enabled various mutations to be described in multiple myeloma. Recent studies have demonstrated that several oncogenic driver mutations were associated with specific types of chromosomal alterations, and increased number of driver mutations was associated with poor outcome.³³ Several groups are investigating the implications of such driver mutations on prognosis and potential therapeutic targets. These techniques will provide new diagnostic and prognostic markers as well as a therapeutic paradigm for multiple myeloma patients.

Radiological Investigations

The purpose of imaging in multiple myeloma is to determine disease involvement of the bone, investigate soft tissue lesions, and assess response to therapy. This is a critical step in diagnosis as well as enabling adequate interventions to prevent fractures and cord compression. Plain radiograph has been the choice of imaging for many years but has now been replaced by other modalities such as low-dose whole body CT, whole body MRI, and PET/CT scans, which provide increased sensitivity and specificity of detecting bone disease.

ADVANCED CONTENT

Plain Radiograph—X-ray Examination Historically, conventional skeletal survey had been the method of choice for evaluation of bony lesions of suspected multiple myeloma cases. Multiple lytic or punched-out lesions are very suggestive of multiple myeloma and are present in 76% of patients at diagnosis (Figs. 23-15 and 23-16). X-ray films of the long bones help identify critical cortical thinning that may lead to pathological fracture, allowing for preventive interventions. However, plain radiography, demonstrating lytic disease only when at least 30% of trabecular bone substance has been lost, provides an inadequate assessment of generalized osteopenia that affects more than 25% of the patients with no lytic disease.

Lytic bone lesions never heal in multiple myeloma, even in patients achieving a complete remission. The use of radiological evaluations such as plain x-ray films for follow-up

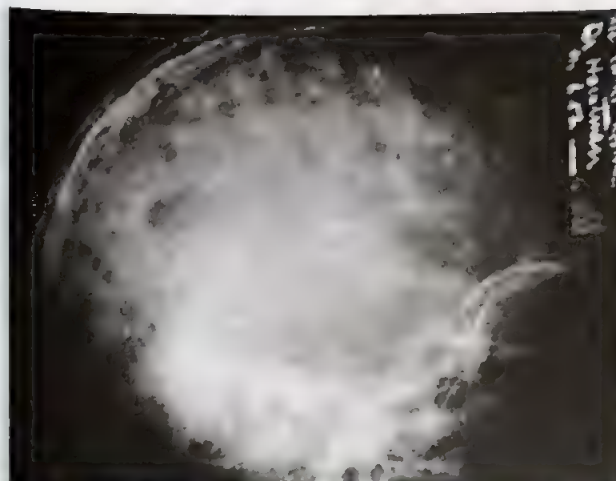


FIGURE 23-15 Extensive lytic skull lesions in a patient with multiple myeloma.



FIGURE 23-16 Radiographic film of the left humerus of a patient with multiple myeloma. Areas with severe cortical bone destruction may be fractured by everyday activities such as lifting or walking (pathological fractures).

is therefore of no value in assessing disease response. However, developing new bone lesions can indicate progressive disease, and any multiple myeloma patients with new pain or neurological symptoms should have repeat appropriate radiological evaluations.

Due to its lack of sensitivity, plain radiographs have fallen out of favor and are reserved for cases when other imaging modalities are contraindicated or unavailable.

Computed Tomography Scanning Computed tomography (CT) scans are more sensitive compared with conventional skeletal survey in detecting lytic bone disease. It further defines suspicious areas on plain films and parts of the skeleton that cannot be evaluated fully by plain films, (e.g., scapulae, ribs, and sternum). Whole body low-dose CT (WBLDCT) allows a valuable study with low radiation dose to the body, is widely accessible, and takes less than a minute to complete. WBLDCT is now one of the preferred front-line imaging modalities for evaluating bone lesions in multiple myeloma.³⁴

Bone Scintigraphy Bone scan has a limited role in multiple myeloma because of the inhibition of osteoblastic activity by the illness.

Magnetic Resonance Imaging Whole body magnetic resonance imaging (MRI) is not only sensitive in detecting bone disease but also evaluates soft tissue and bone marrow compartment. It is also the imaging of choice when evaluating the central nervous system (Fig. 23-17). The ability to evaluate the bone marrow is valuable as it is often focally involved in multiple myeloma, and the percentage of plasma cells in the bone marrow is important in both the diagnosis and response assessment to therapy.



FIGURE 23-17 MRI showing compression fracture at L-2 (red arrow) and focal plasmacytoma (white arrows and circles).

Image-guided biopsy and random bone marrow biopsy often reveal discordant plasma cell percentage. The major limiting factor in its implementation into everyday practice is that it is not widely available. Thus, whole body MRI could be considered in suspected cases of myeloma with equivocal WBLDCT and/or PET/CT when evaluating for cord compression or in solitary plasmacytoma. It can also be obtained when evaluating response to therapy or detecting early relapse.³⁴

Positron Emission Tomography Positron emission tomography (PET) imaging with 18-fluorine-fluoro-deoxyglucose (FDG) has emerged as a new scanning technique in multiple myeloma. PET scan is very sensitive and specific in detecting bone disease, bone marrow involvement, and extramedullary disease (Fig. 23-18). PET scan can also distinguish between metabolically active disease and inactive disease. Changes in FDG activity provide valuable information about response to therapy and is the preferred modality for this purpose. Given these advantages, PET/CT is one of the preferred imaging modalities for initial diagnosis of multiple myeloma, diagnosis of extramedullary plasmacytoma, and assessment of response to therapy.³⁵

Dual Energy X-ray Absorptiometry Scanning Dual energy x-ray absorptiometry scanning (DEXA) is the standard modality for diagnosing osteoporosis. In multiple myeloma patients, low-lumbar spine bone mineral density at diagnosis is correlated with an increased risk of vertebral collapse and fractures. It is an important test to consider, especially if there is no lytic bone disease. Follow-up imaging is not routinely indicated in multiple myeloma.

Diagnostic Criteria

Once the diagnosis of multiple myeloma is suspected based on the presence of symptoms such as anemia, renal insufficiency, bone pain, neuropathy, and by the presence of high protein levels, confirmatory testing is needed to determine the stage and prognosis. Box 23-2 outlines the components of evaluating patients with newly diagnosed multiple myeloma.

Plasma cell neoplasm encompasses a spectrum of disease, from MGUS, in which the level of monoclonal protein is low and does not cause any clinical significance, to smoldering myeloma (which is an intermediate stage), to multiple myeloma, in which patients present with symptomatic and potentially aggressive disease. The current diagnostic criteria are listed in Table 23-4.³⁶ A clonal plasma cell population in the bone marrow or a plasmacytoma must be present. The key distinguishing feature of multiple myeloma from MGUS and smoldering myeloma is the presence of end-organ damage caused by the monoclonal protein, abbreviated as the CRAB criteria (hyperCalcemia, Renal failure, Anemia, Bone lesions). Even if the CRAB criteria are not met, the high-risk smoldering myeloma patients (>60% clonal plasma cells in the bone marrow or ratio of the involved to the uninvolved



FIGURE 23-18 PET scan showing metabolic activity in multiple plasmacytomas throughout the skeleton.

serum light chain >100) suggest a very high probability of progression and will benefit from early initiation of treatment. This is now reflected in the current diagnostic guidelines.

The differential diagnosis of multiple myeloma includes related plasma cell disorders such as MGUS, smoldering myeloma, light-chain amyloidosis, and other lymphoproliferative disorders such as Waldenström macroglobulinemia and non-Hodgkin's lymphoma.

Staging

Once a diagnosis of multiple myeloma has been established, the stage of the disease can provide valuable information on prognosis. Staging is also important for reporting treatment outcomes and provides a common means of comparing

clinical trials. The Durie and Salmon staging has been used historically but has been replaced by the International Staging System (ISS).

The ISS was developed based on 10,750 previously untreated multiple myeloma patients from 17 institutions worldwide (Table 23-5). It is based on the levels of serum β_2 M and serum albumin and classifies patients into three stages. Patients who are stages I, II, and III had median survival of 62 months, 44 months, and 29 months, respectively.³¹

Other important prognostic factors including chromosomal abnormalities and LDH have been recognized and are now incorporated into the Revised International Staging System (R-ISS) (Table 23-6).³² In addition to β_2 M ≥ 5.5 mg/L (which would put the patient in ISS stage III), presence of either t

BOX 23-2 Evaluation of Patients With Newly Diagnosed Multiple Myeloma**M-Protein in Serum**

- Electrophoresis and immunofixation
- Quantitation of immunoglobulins
- Serum viscosity if symptomatic
- Serum free light chain

24-Hour Urine for Urinary Protein Electrophoresis (UPEP)/IFE (Immunofixation) and Creatinine Clearance**Bone Marrow Aspirate and Biopsy**

- Cytogenetics/FISH
- Flow cytometry (plasma cell labeling index)

CBC With Reticulocyte and Differential**Chemistry Panel**

(Renal, Ca, Albumin, Uric Acid)

 β_2 -Microglobulin, C-Reactive Protein, and LDH**Radiological Evaluation**

- Skeletal survey
- MRI (spine, pelvis, and skull)
- PET/CT scan
- Bone densitometry

Organ Function

- Echocardiogram
- ECG
- Pulmonary function tests
- Viral testing

TABLE 23-4 Diagnostic Criteria for Multiple Myeloma

- I. Biopsy-proven plasmacytoma or
- II. Clonal bone marrow plasmacytosis ($\geq 10\%$) and any one of the following:
 - i. Myeloma defining events:
 - Hypercalcemia; >11 mg/dL or >1 mg/dL higher than upper limit of normal
 - Renal; creatinine clearance <40 mL/min or creatinine >2 mg/dL
 - Anemia; Hgb <10 g/dL or <2 g/dL below the lower limit of normal
 - Bone; one or more lytic lesion on skeletal radiography, CT, or PET/CT
 - ii. Biomarkers of malignancy:
 - Clonal bone marrow plasma cells $\geq 60\%$
 - Involved to uninvolved serum light-chain ratio ≥ 100
 - >1 focal lesions on MRI study

Source: Adapted from Rajkumar SV, et al. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. *Lancet Oncol*. 2004;15:e538.

LDH or high-risk chromosomal abnormalities would classify the patient into stage III in the R-ISS staging system. High-risk chromosomal abnormalities were defined as deletion of chromosome 17p, t(4;14), or t(14;16). These patients exhibited worse prognosis with 5-year survival of 40%. R-ISS

TABLE 23-5 International Staging System

Stage	Criteria	Median Survival (months)
I	Serum β_2 -microglobulin <3.5 mg/L and serum albumin ≥ 3.5 g/dL	62
II	Not stage I or III	44
III	Serum β_2 -microglobulin ≥ 5.5 mg/L	29

There are two categories for stage II: serum β_2 -microglobulin <3.5 mg/L but serum albumin <3.5 g/dL; or serum β_2 -microglobulin 3.5 to <5.5 mg/L irrespective of the serum albumin level.

Source: Adapted from Grippie PR, et al: International staging system for multiple myeloma. *J Clin Oncol*. 2005;23:3412.

TABLE 23-6 Revised International Staging System (R-ISS)

Stage	Criteria	5-Year Survival (%)
I	ISS stage I, normal LDH, and standard-risk chromosomal abnormalities	82
II	Not R-ISS stage I or III	62
III	ISS stage III and either high LDH or high-risk chromosomal abnormalities (del(17p), t(4;14), or t(14;16))	40

Source: Adapted from Palumbo A, et al: Revised International Staging System for Multiple Myeloma. *J Clin Oncol*. 2015;33:2863.

should be used to stage all new patients diagnosed with multiple myeloma.

Treatment

There have been many exciting developments in myeloma treatment that have significantly improved prognosis. The treatment for multiple myeloma is broadly categorized as induction therapy with a goal of achieving deep response, consolidation therapy with autologous hematopoietic transplantation (HCT) in those who are eligible, maintenance therapy to improve the depth and duration of response, and supportive care that addresses the organ dysfunction and symptoms caused by the clonal proliferation of plasma cells or the monoclonal proteins.

Induction Therapy

The goal of induction therapy is to rapidly control the disease to minimize the complications caused by multiple myeloma, such as renal failure, hypercalcemia, and compression of the spine, to induce a deep response and minimize the toxicity caused by the therapy. Multiple studies have shown that a deep response translates into improved progression-free survival (PFS) and overall survival (OS).¹⁹ Although current first-line regimens do not significantly affect the ability to collect

cells, it is important to determine whether the patient will be eligible for consolidation autologous transplant and to prepare accordingly.

ADVANCED CONTENT

Multiple randomized controlled trials have shown the superiority of triplet therapy compared with doublet therapy. Thus, the standard first-line therapy for newly diagnosed multiple myeloma should be triplet combination therapy except for special circumstances such as poor expected tolerance to therapy. Bortezomib, lenalidomide, and dexamethasone (VRd) is a widely used first-line therapy in newly diagnosed multiple myeloma in both transplant eligible and ineligible patients due to high efficacy and low toxicity. In the phase 3 randomized controlled study SWOG S0777, VRd was compared with lenalidomide and dexamethasone (Rd) followed by Rd maintenance in both groups. The triplet therapy had significant improvement of both median PFS (41 vs. 29 months) and median OS (not reached vs. 69 months) at median follow-up of 84 months.⁴⁰

Another option for front-line therapy is the combination of cyclophosphamide, bortezomib, and dexamethasone (CyBorD). Data from phase II EVOLUTION study have shown 75% overall response rates and 1-year PFS of 93% in patients who were treated with CyBorD. Notably, the 5-year PFS was 42% and OS was 70%.⁴¹ CyBorD is one of the preferred regimens in newly diagnosed patients with acute renal failure, with consideration of switching to VRd if the kidney function improves.

Combination treatments with daratumumab, an anti-CD38 monoclonal antibody, are also an attractive option. Daratumumab has been combined with lenalidomide and dexamethasone (DRd) in transplant ineligible patients in the MAIA study. A total of 737 patients were randomly assigned to either DRd or Rd (control arm). The PFS at 30 months was 70.6% in the DRd arm and 55.6% in the control arm. Minimal residual disease (MRD) negativity was achieved in 24.2% of the daratumumab arm and 7.3% in the control arm.⁴² Based on these results, DRd is an excellent first-line option for patients who are ineligible for autologous HCT. Of note, daratumumab causes panagglutination on indirect antibody testing by binding to CD38 on RBCs; thus, RBC typing is routinely done before initiation of anti-CD38 monoclonal antibodies.⁴³

Four-drug combination for front-line therapy has been studied in the GRIFFIN trial, in which 207 patients with newly diagnosed multiple myeloma were randomized into induction with VRd with or without daratumumab, followed by autologous HCT and two additional cycles of the induction regimen post-transplant, then maintenance with lenalidomide with or without daratumumab. With median follow-up of 22.1 months, the stringent complete response rate was 62.6% in the daratumumab arm and 45.4% in the control arm. More infections were observed in the daratumumab arm, but the grade 3/4 infections were comparable.⁴⁴ Long-term follow-up is ongoing.

Consolidation With Autologous Hematopoietic Cell Transplantation (HCT)

All patients who meet the criteria for transplant should be referred to a transplant center for evaluation. In the first randomized study in 1996 by Attal et al., high-dose chemotherapy with autologous HCT demonstrated higher response rates, event-free survival, and OS compared with conventional therapy.⁴⁵ Since then, autologous HCT has been a standard part of therapy for patients with multiple myeloma. Various randomized studies have demonstrated the benefit of autologous HCT in prolonging the PFS, but the survival benefit is debated.

ADVANCED CONTENT

The IFM 2009 study randomized 700 patients to either three cycles of VRd induction followed by either high-dose melphalan autologous HCT and three cycles of post-transplant VRd or five additional cycles of VRd. The median PFS was 50 months in the transplant group and 36 months in the control group. CR was achieved in 59% of the transplant arm and 48% in the control arm. At median follow-up of 4 years, OS was 81% in the transplant arm and 82% in the control arm.⁴⁶

Autologous transplant has an advantage of providing prolonged period without disease and deep response, which may translate into a survival benefit with longer term follow-up. At the time of initial stem cell collection, enough cells for a second autologous HCT should be collected as a second transplant may be considered for carefully selected patients who had a durable response to the first transplant.⁴⁷

Maintenance Therapy

Several studies have investigated the concept of maintenance therapy after the initial induction therapy. Lenalidomide has been an attractive maintenance agent that is efficacious and well tolerated orally. Although multiple randomized control studies have demonstrated PFS benefit, individual studies have not demonstrated OS benefit, likely due to short follow-up.

ADVANCED CONTENT

A recent meta-analysis of lenalidomide maintenance therapy after autologous HCT demonstrated that at median follow-up of 79.5 months, median OS was not reached in the lenalidomide arm and was 86.0 months in the placebo or observation group.⁴⁸ Note that treatment with lenalidomide led to increased risk of secondary malignancies (6% to 7% absolute risk) and increased neutropenia. Bortezomib or ixazomib maintenance can be considered for patients who are not candidates for lenalidomide maintenance, or those with high-risk disease with 17p deletion.⁴⁹

Relapsed Disease

There has been tremendous advancement in the treatment of multiple myeloma in the last decade. Multiple agents have been approved for the use of relapsed or refractory disease and more are under investigation. Second generation proteasome inhibitor carfilzomib and oral proteasome inhibitor ixazomib can be combined with various classes of drugs in those who have progressed after bortezomib. Pomalidomide, a third-generation immunomodulatory drug (IMiDs), is an alternative to lenalidomide. Other newly approved treatments include anti-B-cell maturation antigen (BCMA) chimeric antigen receptor (CAR) T-cell therapy, belantamab mafodotin-blmf (antibody-drug conjugate targeting BCMA), elotuzumab (signaling lymphocytic activation molecule family (SLAMF7) inhibitor), selinexor, venetoclax (BCL2 inhibitor), and panobinostat (histone deacetylase inhibitor). An excellent review by Rajkumar summarizes the new advancements in the treatment of multiple myeloma and would be of interest for advanced learners.⁵¹

Supportive Therapy

Bone-modifying agents have shown benefit in decreasing skeletal complications and prolonging PFS regardless of bone involvement of the multiple myeloma. Bisphosphonates such as pamidronate and zoledronic acid have long been used. A recent meta-analysis demonstrated decreased pathological vertebral fractures and skeletal-related events with treatment with bisphosphonates. Treatment with zoledronic acid was also associated with increased overall survival.⁵² Receptor activator of nuclear factor kappa-B ligand (RANKL) inhibitor denosumab has also been studied in patients with newly diagnosed multiple myeloma. In a large phase III randomized controlled trial of 1,718 patients, those with newly diagnosed multiple myeloma with at least one lytic bone lesion were randomized to either zoledronic acid or denosumab every 4 weeks. Denosumab was found to be noninferior to zoledronic acid for time to first skeletal event.⁵³ Based on these studies, zoledronic acid or denosumab should be initiated in all patients with multiple myeloma who are undergoing treatment. Note that osteonecrosis of the jaw is a rare but serious complication of these treatments, and patients should receive dental evaluation before initiation of these treatments.

Radiation therapy has limited efficacy in treating systemic multiple myeloma. However, it is highly effective in treating the localized disease process such as symptomatic bone lesions or plasmacytoma. Kyphoplasty or vertebroplasty can be considered for painful vertebral compression fractures.

Hypercalcemia is managed by intravenous saline and medications that inhibit osteoclasts, such as glucocorticoids, bisphosphonates, and calcitonin.

In patients with recurrent infections, intravenous pooled immunoglobulin given at monthly intervals may be of significant benefit, especially if the IgG level is less than 500 mg/dL. In addition, EPO could be considered to maintain adequate hemoglobin levels.

Variants of Plasma Cell Syndromes

Solitary Plasmacytoma

Solitary plasmacytoma presents with a single plasmacytoma either in the bone (also known as solitary plasmacytoma of bone) or soft tissue (also known as extramedullary plasmacytoma). Diagnosis requires a biopsy to confirm the presence of a monoclonal plasma cell infiltrate.³⁶ When there is <10% clonal plasma cells in the bone marrow along with the single plasmacytoma, it is termed solitary plasmacytoma with minimal marrow involvement. These patients, by definition, do not have any evidence of end-organ damage (no other lytic lesions, normal calcemia, absence of anemia, normal renal function). It is thus important to thoroughly investigate for other plasmacytomas or systemic symptoms. The tumor may secrete immunoglobulin, resulting in a small serum M-spike that usually disappears after therapy. Treatment is radiation therapy to the plasmacytoma without systemic therapy. Patients should undergo surveillance as the risk of progression in 3 years is approximately 10% for solitary plasmacytoma of soft tissue, 20% for solitary plasmacytoma of soft tissue with minimal marrow involvement, and 60% for solitary plasmacytoma of bone with minimal marrow involvement.³⁶

Plasma Cell Leukemia

Plasma cell leukemia (PCL) is a rare variant of multiple myeloma (2% to 3%), with an aggressive presentation and short survival. It is defined as circulating peripheral blood plasma cells exceeding 2,000/ μ L, and 20% of peripheral blood white blood cells (Fig. 23-19). Half of these cases are found in advanced multiple myeloma, usually late in the course of the disease, and usually heralding a terminal event. The other half, often referred to as primary plasma cell leukemia, affects newly diagnosed patients. Patients present with high calcium levels, kidney failure, and often more severe anemia, thrombocytopenia, and involvement of various organs, such as the liver, spleen, and lymph nodes. Prognosis is poor, with



FIGURE 23-19 Peripheral blood in plasma cell leukemia showing presence of circulating plasma cells. (From Dutcher, T. Hematology. In: Look, and Learn. Health and Education Resources, Inc: Bethesda, MD with permission.)

expected survival of only several months, even after autologous and/or allogeneic transplantation.⁵⁴

Nonsecretory Myeloma

Fewer than 1% of multiple myeloma patients present with no abnormal immunoglobulin in the blood or urine, even with sensitive assays. These patients present with lytic bone disease with elevated serum calcium and clonal plasma cells on bone marrow biopsy. Because of normal levels of immunoglobulin, infectious complications are fewer. The absence of light-chain excretion through the kidneys preserves renal function, and survival is often better than that associated with other multiple myeloma subtypes.

POEMS Syndrome

Polyneuropathy, organomegaly, endocrinopathy, M-protein, skin changes (**POEMS**) syndrome is a paraneoplastic syndrome related to underlying plasma cell dyscrasia. The pathophysiology of this disease is poorly understood. Diagnosis is often delayed due to its rarity and overlapping signs and symptoms with other systemic diseases. Patients must have polyradiculoneuropathy and monoclonal gammopathy as part of the major criteria. Other presenting symptoms include sclerotic bone lesion, volume overload, thrombocytosis, and polycythemia. The mainstay of therapy is irradiation of the dominant sclerotic plasmacytoma. Systemic therapy may also be needed.⁵⁵

Waldenström Macroglobulinemia

Waldenström macroglobulinemia (WM) is an indolent B-cell lymphoma in which the malignant B cells exhibit plasma cell differentiation (plasmacytoid lymphocytes). These cells produce IgM monoclonal protein. WM cells express pan-B lymphocyte surface antigens (CD19, CD20, and CD24) with light-chain restriction (mostly κ). Recurrent mutations have been described in WM. Notably, more than 90% of cases of WM display myeloid differentiation primary response 88 (MYD88) mutation, and 30% to 40% have C-X-C motif chemokine receptor 4 (CXCR4) mutation.⁵⁶ The bone marrow is extensively infiltrated with lymphoid and plasmacytoid cells. Intranuclear vacuoles containing IgM monoclonal protein (**Dutcher bodies**) within the malignant cells of WM are common (Fig. 23-20).

In the United States, WM affects 1,400 individuals each year. The median age at diagnosis is 64 years and has a male predominance. There is a clear familial clustering in families that can be seen in as many as 20% of the cases.

Patients with WM generally present with fatigue, weight loss, and generalized weakness. Other symptoms are related to the IgM paraprotein produced by the malignant cells and include cryoglobulinemia, autoimmune hemolytic anemia, and hyperviscosity (see previous section on hyperviscosity syndrome). Patients also experience coagulopathy, can have extensive bruising (cryoglobulinemic purpura), and may have bleeding from the gums and nose (Fig. 23-21). More severe platelets and blood clotting factor proteins by the abnormal

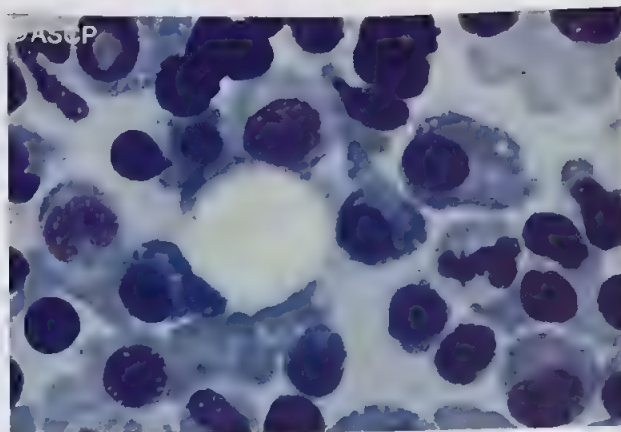


FIGURE 23-20 Plasmacytoid lymphocytes in marrow aspirate from a patient with Waldenström macroglobulinemia. (From Hyun, BH. Morphology of Blood and Bone Marrow. American Society of Clinical Pathologists. Workshop 5121, Philadelphia, September 7, 1983, with permission.)

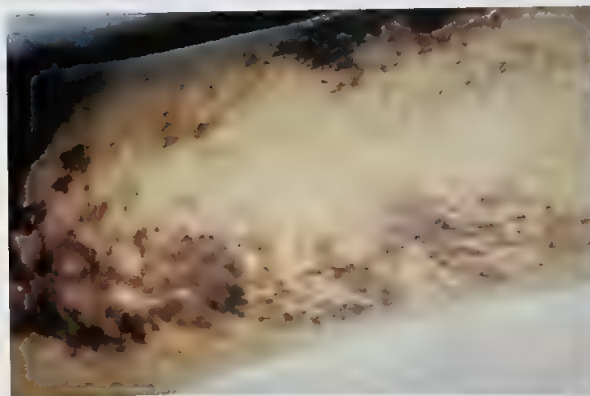


FIGURE 23-21 Arm of patient with cryoglobulinemic purpura. Note the skin manifestations.

IgM. Tissue infiltration with the malignant cells or deposition of IgM or amyloid fibrils may produce cytopenias, lymphadenopathy, or hepatosplenomegaly. Anemia is the main feature in most symptomatic WM patients (80%). Progressive sensory motor peripheral neuropathy is another presenting feature of this disease.

The diagnosis of WM is made by demonstrating IgM paraprotein and $\geq 10\%$ bone marrow involvement with plasmacytoid lymphocytes. MYD88 mutation, when present, can help distinguish WM from other indolent lymphomas. It is also important to differentiate WM from IgM MGUS or multiple myeloma. While WM presents and behaves more similarly to an indolent lymphoma, IgM multiple myeloma should meet the diagnostic criteria of multiple myeloma.

Similar to other indolent lymphomas, treatment can be delayed until patients develop symptoms from the disease. Common indications for treatment are systemic symptoms (fever, night sweats, weight loss), organ compromise from lymphadenopathy or hepatosplenomegaly, cytopenias, and symptoms of hyperviscosity.

The treatment of WM is with rituximab-based regimens. Rituximab, an anti-CD20 monoclonal antibody, can be combined with alkylating agents (bendamustine, cyclophosphamide), proteasome inhibitors (bortezomib, carfilzomib, ixazomib), or bruton kinase inhibitor (BTK) ibrutinib for first-line therapy. Ibrutinib alone can also be considered for first-line therapy.⁵⁷ If severe symptoms are present at diagnosis, immunoglobulins may be quickly removed by plasmapheresis, resulting in the rapid improvement in symptoms.⁸

CRITICAL THINKING QUESTION

23-4 Why does the viscosity of samples collected from patients with Waldenström macroglobulinemia increase when left at room temperature?

Light-Chain Amyloidosis

Amyloidosis is a rare and potentially fatal disease that occurs when insoluble protein fibrils are deposited in tissues and organs, impairing their function (e.g., heart, kidneys, liver, spleen, nervous system, and gastrointestinal tract) (Fig. 23-22). There are many precursor proteins that may result in amyloidosis. The most common is light-chain (AL) amyloid (amyloid with presence of light chain secretion); the precursor protein is derived from immunoglobulin light-chain fragments and is associated with a clonal plasma or B-cell proliferative disorder. Less common is AA amyloidosis (secondary amyloidosis associated with inflammation). This usually complicates chronic diseases in which there is ongoing or recurring inflammation, such as rheumatoid arthritis or spondyloarthropathy, chronic infections, or periodic fever syndromes. It is important to distinguish between the different types of amyloidosis as the treatment is different. Here, we limit our discussion to AL amyloidosis.

For diagnosis, patients must have histopathologic evidence of AL amyloid protein deposition in the organs. The kidney (70%), heart (60%), liver (60%), gastrointestinal tract (30%), and peripheral nerves (15%–20%) can be involved. Resulting symptoms include nephrotic range proteinuria and renal failure, congestive heart failure, hepatomegaly, gut dysmotility, and peripheral neuropathy. Coagulopathy is also commonly seen, and acquired von Willebrand disease could be present. Patients demonstrate free lambda light chain in 70% of cases and free kappa light chain in 25% of cases. Intact immunoglobulin is only rarely found. Approximately half of the clones have $\kappa(11;14)$ and 20% have gain of $1(q21)$.⁵⁸ The revised Mayo Clinic Amyloid Staging system gives prognostic information, from overall survival of 94 months for stage I disease to 6 months for stage IV disease.⁵⁹

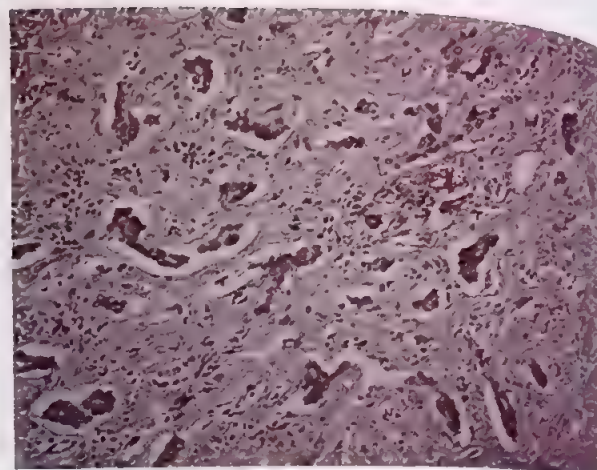


FIGURE 23-22 Amorphous amyloid deposits replacing normal liver architecture.

Treatment often is aimed at eliminating the source of the abnormal precursor protein and limiting further organ damage. Daratumumab in combination with CyBorD is the first line of therapy based on the ANDROMEDA trial that demonstrated significantly higher organ response rates and PFS with the addition of daratumumab to CyBorD.⁶⁰ Autologous HCT can be considered in low-risk patients whose organ functions are relatively preserved.

Light-Chain Deposition and Heavy-Chain Diseases

Light-chain deposition disease (LCDD) is similar to primary amyloidosis; both are clonal plasma cell proliferative disorders, but the light-chain fragments do not form amyloid fibrils, and while most cases of AL-amyloidosis are related to lambda secretion, LCDD is usually kappa. As with AL-amyloidosis, LCDD may be associated with multiple myeloma or other conditions, such as lymphoma or Waldenström macroglobulinemia. In the kidney, LCDD is often associated with renal failure, in contrast to AL-amyloidosis, which results in excess protein excretion. Treatment is directed at eliminating the plasma cell clone. If the patient is young and has end-stage renal disease, renal transplants may be considered.

Heavy-chain diseases (HCDs) are very heterogeneous groups of disorders and do not represent true plasma cell disorders. They are mentioned here owing to the presence of abnormal serum immunoglobulin components. The B cells in these disorders exclusively produce monoclonal heavy chains and no light chains. Patients have systemic symptoms and organomegaly. The details of these disorders are beyond the scope of this chapter.

SUMMARY CHART

- Plasma cells contribute to humoral immunity by producing immunoglobulins.
- Immunoglobulins have two heavy chains and two light chains.
- Immunoglobulin heavy chains are alpha, delta, epsilon, mu, and gamma. Light chains are kappa and lambda.
- Monoclonal gammopathy of undetermined significance (MGUS) is evident when a patient has a small M spike, urine M-protein of less than 500 mg per 24 hours, no lytic bone lesions, less than 10% plasma cells in the bone marrow, and no end-organ damage from the monoclonal protein overproduction.
- The intermediary stage of MGUS and multiple myeloma is smoldering myeloma and is evident with the patient has a M spike of >3 g/dL, urine M-protein levels of >500 mg per 24 hrs, and no end-organ damage.
- Multiple myeloma is a disorder characterized by:
 - Clonal proliferation of abnormal plasma cells in the bone marrow that produces antibodies referred to as monoclonal gammopathy.
 - *Plasmacytoma*, a tumor of plasma cells.
 - Bence-Jones proteins secreted in the urine that precipitates at 56°C.
 - Patients present with bone destruction, hypercalcemia, kidney failure, and hyperviscosity as well as pancytopenia, causing anemia, bleeding diathesis, and increased susceptibility to infection.
- Laboratory evaluation in multiple myeloma includes electrophoresis, immunofixation, quantitative immunoglobulin assay, complete blood count (CBC), peripheral blood smear, erythrocyte sedimentation rate (ESR), bone marrow biopsy examination, chemistry panel, beta₂ microglobulin (β₂M), lactate dehydrogenase (LDH), and C-reactive protein (CRP). Diagnostic procedures include whole body low-dose computed tomography (CT), positron emission tomography (PET), and magnetic resonance imaging (MRI).
- Staging in multiple myeloma provides an estimated extent of disease to help determine the appropriate treatment plan and patient prognosis.
- IL-6 and osteoclast-activating factor (OAF) play major roles in the production of the lytic bone lesions associated with multiple myeloma by stimulating the development of osteoclasts and subsequent release of calcium from bone, thereby weakening the bone structure.
- Three-drug combination therapy is the primary treatment in multiple myeloma.
- Variants of plasma cell syndromes include solitary plasmacytoma, plasma cell leukemia, and POEMS.
- Waldenström macroglobulinemia is an indolent B-cell lymphoma with overproduction of monoclonal IgM antibodies by *plasmacytoid lymphocytes*.
- Manifestations associated with Waldenström macroglobulinemia include IgM monoclonal protein, hyperviscosity syndrome, fatigue, cryoglobulinemic purpura, and bleeding diathesis.
- Standard treatment in Waldenström macroglobulinemia includes rituximab-based therapy and plasmapheresis to remove increased levels of IgM.
- AL amyloidosis occurs in multiple myeloma when immunoglobulin kappa (κ) or lambda (λ) light chains deposit in organs such as the heart, kidneys, nerves, liver, spleen, and gastrointestinal tract.

CASE STUDY 23-1

A 65-year-old man presented with a 6-month history of back pain. His wife noted that he was frequently confused, slept much of the day, and made occasional nonsensical statements. He does not have any other medical comorbidities and is otherwise fit. On evaluation, his hemoglobin (Hgb) level was 9.5 g/dL with a mean corpuscular volume (MCV) of 91 fL. He had a calcium level of 10.6 mg/dL (normal range is 8.8 to 10.5) with albumin level of 2.6 g/dL and a corrected calcium of 11.7 g/dL. The creatinine level was 1.1 g/dL. The patient was hydrated with normal saline and received bisphosphonate infusion (zoledronic acid) that rapidly lowered the calcium levels, and the patient's mental status quickly improved over the following 3 days. Serum protein electrophoresis revealed an M-spike measuring

4.7 g/dL. Immunoelectrophoresis revealed that the M-spike was composed of an IgG-κ antibody. His serum IgM level was 63 mg/dL, and the IgA level was 120 mg/dL. The β₂ microglobulin was 3.4 mg/dL and LDH was 200 IU/L. A 24-hour urine collection demonstrated no monoclonal protein. The bone marrow was hypercellular with 70% plasma cells, which had kappa restriction. Cytogenetics and FISH studies showed normal male karyotype. A low-dose CT scan revealed lytic lesions in the skull, pelvis, both arms, and both femurs.

QUESTIONS

1. What diagnostic criteria for multiple myeloma are met in this patient?

Continued

CASE STUDY 23-1—cont'd

2. What R-ISS stage myeloma does he have?
3. What is the prognosis of this patient?
4. Describe the standard therapies that could be used for this patient.

ANSWERS

1. On bone marrow biopsy, he has clonal plasma cells with kappa restriction. He meets the CRAB criteria with anemia, hypercalcemia, and lytic bone lesions.
2. The patient has R-ISS stage II (β_2 microglobulin <3.5 mg/L, serum albumin <3.5 g/dL, standard risk cytogenetics and FISH, and normal LDH).
3. The prognosis of R-ISS stage II MM is 5-year survival of 62% (see Table 23-6).
4. Since he does not have major medical comorbidities and is otherwise healthy, he appears to be eligible for autologous HCT. The standard therapy will be 3-drug combination induction, most commonly with bortezomib, lenalidomide, and dexamethasone (VRd), autologous HCT consolidation, followed by maintenance therapy. The patients should receive bisphosphonates to prevent skeletal complications such as fractures.

CASE STUDY 23-2

A 55-year-old man presented with elevated gamma gap on routine blood work. He is otherwise healthy and does not complain of any fever, chills, night sweats. On physical exam, he did not have any lymphadenopathy, hepatosplenomegaly, or focal bony tenderness. Serum protein electrophoresis revealed an M-spike of 1.6 g/dL. Immunofixation identified the M-spike as IgA-lambda. Free light-chain ratio was 0.40, and 24-hour urine collection for protein evaluation showed no monoclonal protein. Serum hemoglobin, creatinine, calcium, albumin, and quantitative immunoglobulins were normal. A whole body low-dose CT did not demonstrate any lytic lesions. The bone marrow biopsy revealed 7% plasma cells with lambda restriction by immunohistochemistry.

QUESTIONS

1. What is the most likely diagnosis for this patient?
 - a. Multiple myeloma
 - b. Waldenström macroglobulinemia
 - c. Monoclonal gammopathy of unknown significance
 - d. Non-Hodgkin's lymphoma
2. What malignancy is most likely to occur in this patient?
 - a. Multiple myeloma
 - b. Chronic lymphocytic leukemia
 - c. Amyloidosis
 - d. Non-Hodgkin's lymphoma
3. What follow-up schedule would be appropriate?
 - a. Monthly serum protein electrophoresis.
 - b. Bone marrow biopsy repeated every 6 months for 2 years. If there is no evidence of myeloma at that time, the patient does not have to return anymore.
 - c. Repeat the complete evaluation (labs, imaging, and bone marrow biopsy) every 6 months.
 - d. Repeat the serum protein electrophoresis in 3 months, then every 6 months thereafter if there has been no change.

ANSWERS

1. c. Although a few patients develop multiple myeloma, non-Hodgkin's lymphoma, or chronic lymphocytic leukemia, most patients with MGUS are not adversely affected by this disorder.
2. a. Multiple myeloma
3. d. Once the initial evaluation is complete, no further testing needs to be done unless there is a change in the production of the M-spike immunoglobulin. Three months is considered a reasonable interval because of the known growth rate of these disorders. If the patient's condition is stable after that time interval, the period of time may safely be increased to 6-month follow-ups for life.

CASE STUDY 23-3

A 59-year-old woman presented with a 35-pound unintentional weight loss over the past 18 months. She had been having trouble concentrating at work, had decreased energy, and had two episodes of blurry vision.

On physical examination, she did not have any enlarged lymph nodes. Her spleen was enlarged and easily felt approximately 7 cm below her left ribs. Her neurological

examination was normal. Blood tests revealed a Hgb of 9.7 g/dL, hematocrit (Hct) of 29.2%, and MCV of 89 dL. Platelet count, white blood cell count, and white cell differential were normal. A chemistry panel was normal with the exception of a total protein level of 9.7 g/dL and albumin of 2.7 g/dL. LDH was 220 IU/L and β_2 microglobulin 3.0 mg/L. The calcium level was 8.3 mg/dL. A serum protein

CASE STUDY 23-3—cont'd

electrophoresis revealed a 5.7 g/dL M-spike. The serum viscosity was 4.1 centipoises.

Immunoelectrophoresis identified the protein as an IgM- κ . The bone marrow was hypercellular with 20% plasmacytoid lymphocytes. MYD88 mutation was detected. She was diagnosed with Waldenström macroglobulinemia.

QUESTIONS

- Which of the following is a possible cause of the patient's problems in concentrating and reading?
 - The calcium level is actually high when the low albumin level is considered.
 - The increased viscosity of the blood is causing poor circulation through the blood vessels in the brain and eyes, resulting in these symptoms.
 - On exposure to cold, cryoglobulins in the patient's head precipitate and block blood vessels.
 - Osteolytic lesions in the spine.
- Which statement is most correct about the patient's prognosis?
 - This disease is rapidly fatal and does not respond to treatment.
 - This is a terminal disease with prognosis of 4 to 5 years.
 - This is a chronic disorder that often requires treatment, and she will probably live another 10 years.
 - She does not need treatment as her disease does not lead to any clinically serious complications.

- If the patient developed more severe neurological problems, what could be done to rapidly lower the IgM in the blood?

ANSWERS

- Hyperviscosity syndrome is common with Waldenström macroglobulinemia, and these are classic symptoms. The plasmacytoid lymphocytes may infiltrate nerves, meninges, and the brain and may be a less common cause of mental status changes and neurological changes. Hypercalcemia is uncommon with Waldenström macroglobulinemia, and correcting the albumin to 4 g/dL (1.3 g/dL increase) would result in a correction of serum calcium level of $0.8 \times 1.3 = 1.04$ mg/dL, totaling 9.34 mg/dL. This is within the normal range. Cooling of the scalp would not result in precipitation of cryoglobulins in the brain but might affect the tips of the ears and nose.
- The prognosis of patients with Waldenström macroglobulinemia is predicted by age, serum LDH, serum albumin, and β_2 microglobulin levels (International Prognostic Scoring System for WM). She has a score of 1 (low-risk) with an expected 10-year survival of 59%.
- Plasmapheresis is useful in removing IgM-rich plasma from the patient within 1 to 2 hours and may result in dramatic resolution of symptoms.

REVIEW QUESTIONS

- Which laboratory test provides the most important prognostic information in multiple myeloma?
 - Lipoprotein Lyase
 - Complete Metabolic Panel
 - β_2 -microglobulin
 - ABO RH Blood Type and Crossmatch
- Which of the following fulfills the diagnostic criteria for multiple myeloma?
 - Biopsy-proven plasmacytoma and 10% to 30% plasma cells in bone marrow
 - More than 30% plasma cells in bone marrow
 - Biopsy-proven plasmacytoma and M-spike on SPEP
 - Monoclonal protein present
- What is a monoclonal spike?
 - Spike on protein electrophoresis caused by monoclonal immunoglobulin
 - A spike in temperature detected on a thermometer
 - Bence Jones Proteins
 - A spike on electrophoresis caused by Hemoglobin S
- Which of the following would be diagnostic criteria for Waldenström macroglobulinemia?
 - IgM M-spike and lymphoplasmacytic cells on bone marrow biopsy
 - Lytic bone lesions and rouleaux
 - Renal failure and more than 30% plasma cells
 - M-spike of IgM, IgG, or IgA; low-normal levels of other immunoglobulins; inability to make light chains
- What does staging refer to?
 - Bone marrow compatibility
 - Estimate of disease severity
 - Chemotherapy regimen
 - Determination of specific immunoglobulin in heavy-chain disease
- Possible renal failure in patients with multiple myeloma are due to which of the following?
 - Heavy chains in the liver
 - Light chains in the spleen
 - Light chain filtration through the kidney
 - Heavy chain deposits in the kidney parenchyma

REVIEW QUESTIONS—cont'd

7. Which of the following is one of the three main causes of complex destruction of various organ systems in multiple myeloma patient?
 - a. Plasma cell count decrease
 - b. Neutropenia as a result of failed bone marrow
 - c. Overproduction of thrombocytes
 - d. Plasma cell expansion
8. What are the common features found on bone marrow biopsy in patients with multiple myeloma?
 - a. Increased numbers of plasma cells with immature binucleated, large cells
 - b. Increased numbers of immature red blood cells with decreased monoblast
 - c. Increased number of megaloblasts
 - d. Decreased number of neutrophils
9. Plasma cells contribute to immunity through which mechanism?
 - a. Phagocytosis
 - b. Antigen identification
 - c. Production of immunoglobulins
 - d. Antigen presentation
10. Which chains listed below are light chains?
 - a. Gamma
 - b. Lambda
 - c. Delta
 - d. Alpha
11. Which immunoglobulin contains five units, making it the largest of the immunoglobulins?
 - a. IgG
 - b. IgM
 - c. IgA
 - d. IgD
12. Which immunoglobulin appears first after exposure to foreign antigen?
 - a. IgA
 - b. IgD
 - c. IgG
 - d. IgM
13. Which immunoglobulin is measurable on reexposure of foreign antigen?
 - a. IgA
 - b. IgD
 - c. IgG
 - d. IgM
14. Which immunoglobulin is involved primarily in allergic/hypersensitivity reactions?
 - a. IgA
 - b. IgE
 - c. IgM
 - d. IgG
15. Confusion, headache, or stroke can occur with which immunoglobulin abnormality?
 - a. Hypogamma globulinemia
 - b. Cryoglobulin production
 - c. Overproduction of immunoglobulins causing hyperviscosity
 - d. Immunoglobulin dysfunction
16. Patients can be susceptible to infection with encapsulated organisms when which condition occurs?
 - a. Hypogamma globulinemia
 - b. Cryoglobulin production
 - c. Overproduction of immunoglobulins
 - d. IgE dysfunction
17. Light chains dumped into the urine are called
 - a. Cryoglobulins
 - b. Gamma globulins
 - c. Bence-Jones proteins
 - d. M-proteins
18. Which of the following findings are present in MGUS?
 - a. Serum M-protein >3 g/dL
 - b. Serum M-protein <3 g/dL
 - c. Urine M-protein >500 mg/24 hrs
 - d. $>10\%$ plasma cells in bone marrow
19. Which of the following findings are present in smoldering myeloma?
 - a. Serum M-protein <3 g/dL
 - b. Urine M-protein >500 mg/24 hrs
 - c. 70% plasma cells in bone marrow
 - d. Evidence of end-organ damage
20. Which demographic is most likely to realize a multiple myeloma diagnosis?
 - a. Adult <50 years old
 - b. Adult <30 years old
 - c. African American adult >60 years old
 - d. African American adults <50 years old
21. Why do patients with multiple myeloma experience increased susceptibility to infections?
 - a. Anemia
 - b. Thrombocytopenia
 - c. Overproduction of cytokines
 - d. Neutropenia
22. Which lab finding would indicate overproduction of immunoglobulins?
 - a. Normocytic, normochromic anemia
 - b. Plasma cells in peripheral blood
 - c. Rouleaux
 - d. Teardrop cells

REVIEW QUESTIONS—cont'd

23. Excessive filtering of immunoglobulins by the kidneys leads to decreased kidney functions. Which lab analysis would indicate if this is happening?
 - a. CBC
 - b. BUN and creatinine levels
 - c. LDH
 - d. ESR
24. The highest circulating plasma cell concentration can be witnessed in which of the following?
 - a. Multiple myeloma
 - b. Solitary plasmacytoma
 - c. Plasma cell leukemia
 - d. POEMS
25. Which immunoglobulin is increased in Waldenström macroglobulinemia?
 - a. IgA
 - b. IgM
 - c. IgG
 - d. IgE
26. Malignant cells in Waldenström macroglobulinemia exhibit which unique finding?
 - a. Dohle bodies
 - b. Flame cells
 - c. Teardrop cells
 - d. Dutcher bodies

See answers at the back of this book.

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Lipid (Lysosomal) Storage Diseases and Histiocytosis

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CHAPTER OUTLINE

Overview of Lipid Storage Diseases

Gaucher's Disease

Historical Perspective
Classification and Clinical Findings
Laboratory Testing and Results
Prognosis
Treatment

Niemann–Pick Disease

Classification and Clinical Findings

Laboratory Testing and Results
Prognosis and Treatment

Tay–Sachs Disease

Clinical Findings
Laboratory Testing and Results
Prognosis and Treatment

Mucopolysaccharidoses

Classification
Clinical Findings

Laboratory Testing and Results
Prognosis and Treatment

Histiocytosis

Sea-Blue Histiocyte Syndrome
Langerhans Cell Histiocytosis

Summary Chart

Case Study 24–1

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 24-1 Identify the appropriate ethnic association with Gaucher's and Tay–Sachs diseases.
- 24-2 Assess the enzyme deficiency seen in Gaucher's disease and the cells where lipids accumulate.
- 24-3 Name the clinical triad seen in Gaucher's disease.
- 24-4 Contrast type I, type II, and type III Gaucher's disease.
- 24-5 Associate appropriate lab analysis and findings with Gaucher's disease.
- 24-6 Describe the appearance of Gaucher's cells.
- 24-7 Identify the enzyme deficiency seen in Niemann–Pick disease.

- 24-8 Correlate Niemann–Pick disease with its associated clinical and laboratory findings.
- 24-9 Identify the enzyme deficiency seen in Tay–Sachs disease.
- 24-10 Describe clinical and laboratory findings associated with Tay–Sachs disease.
- 24-11 Evaluate the clinical findings present in mucopolysaccharidoses.
- 24-12 Assess relevant laboratory analysis and results for mucopolysaccharidoses.
- 24-13 Describe the characteristic cell associated with sea-blue histiocyte syndrome.

This chapter explains lipid storage diseases, which are rare, autosomal inherited disorders. Also known as *lysosomal storage diseases*, there is subcellular accumulation of unmetabolized material in the lysosomes of various cells. Lipid, or lysosomal, storage diseases are caused by various enzyme defects (inborn errors) in lipid metabolism that are linked to an enzyme deficiency. Although many different types of lipid storage disorders have been documented, the most widely known and well-established diseases are Gaucher's, Niemann–Pick, Tay–Sachs, and mucopolysaccharidoses.

Overview of Lipid Storage Diseases

Although all ethnic groups are known to be affected by lipid storage diseases, there is an increased incidence of selected disorders such as Gaucher's and Tay–Sachs diseases in certain

ethnic groups, most notably Ashkenazi Jews (Jews who trace their origin to the Baltic Sea region).

Lipid storage diseases have a wide clinical expression, ranging from patients who are essentially asymptomatic to those with severe and incapacitating signs and symptoms with early death.

The aim of treatment for these disorders has been directed at prenatal detection. The only currently effective, proven therapy is enzyme replacement, which represents a new type of treatment for genetic disorders that has improved the lives of many patients. The greatest controversy regarding enzyme replacement therapy is the optimum amount and frequency of treatment.¹ In addition, hematopoietic stem cell transplantation is an effective treatment for various lipid storage disorders.² Bone marrow transplantation is an extremely aggressive, expensive, and high-risk therapy, in contrast to the safe and practical treatment of enzyme replacement therapy.

general characteristics of lysosomal storage diseases are summarized in Box 24-1.

Gaucher's Disease

Historical Perspective

Gaucher's disease was first described in 1882 by Philippe C. Gaucher in a 32-year-old woman with an enlarged spleen. Gaucher believed that the abnormal cells found in her spleen at autopsy were part of a primary splenic tumor. This abnormal cell, later named *Gaucher's cell*, is the result of the deficiency of the enzyme **beta (β)-glucocerebrosidase**, which leads to an accumulation of unmetabolized substrate glucocerebroside in cells, predominantly the monocyte-macrophage system (the reticuloendothelial system; Fig. 24-1). Gaucher's observations were studied further, and the entity known as Gaucher's disease was defined and characterized as a familial disorder at the turn of the last century.³ In 1920, another variation or type of Gaucher's disease characterized by neurological involvement was first described. It was after this date that Gaucher's disease was classified as a lysosomal storage disorder resulting from an enzyme deficiency with an

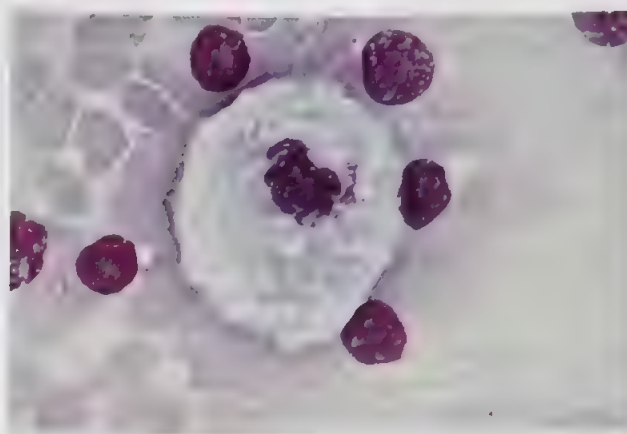


FIGURE 24-1 Gaucher's cell, bone marrow aspirate.

autosomal-recessive inheritance pattern. Although Gaucher's disease is the most frequent lysosomal storage disease, it was not until 1965 that the actual enzyme deficiency was identified as glucocerebrosidase. In 1984, the gene for glucocerebrosidase was cloned, and in 1991, an important breakthrough occurred with the initiation of clinical trials for enzyme replacement therapy at the National Institutes of Health (NIH).³ Gaucher's disease became the first enzyme-deficiency disorder to be successfully treated with infusion of replacement enzyme.⁴

Classification and Clinical Findings

Gaucher's disease has three clinically recognizable subtypes:

- Type I – nonneuronopathic (adult form)
- Type II – acute neuronopathic (infant form)
- Type III – subacute neuronopathic (juvenile form)

Gaucher's types I, II, and III have in common the clinical triad of hepatosplenomegaly, the finding of Gaucher's cells in the bone marrow, and an increase in serum acid phosphatase.³ The severity of the disease and the patient's age when the disease first manifests are related to the magnitude of the enzyme deficiency. The clinical findings of each type of Gaucher's disease are briefly summarized in Table 24-1.

BOX 24-1 General Characteristics of Lipid Storage Diseases

- Rare, inherited autosomal-recessive disorders
- Also known as lysosomal storage diseases because of accumulation of unmetabolized material in lysosomes
- Caused by enzyme deficiencies in lipid metabolism
- Increased incidence of some lipid storage diseases in certain ethnic groups (i.e., Gaucher's disease in Ashkenazi Jews)
- Great variation in clinical expression (i.e., asymptomatic to severe with early death)
- Effective therapy: enzyme replacement
- Most well-known and characterized: Gaucher's disease, Niemann-Pick disease, Tay-Sachs disease, and mucopolysaccharidoses

TABLE 24-1 Gaucher's Disease: Clinical Findings by Subtype

Clinical Findings	Subtype		
	Type I: Nonneuronopathic (Adult Form)	Type II: Acute Neuronopathic (Infant Form)	Type III: Subacute Neuronopathic (Juvenile Form)
Clinical onset	Childhood/adulthood	Infancy	Childhood/juvenile
Estimated frequency	1/450–1/1,000	1/100,000	1/100,000
Hepatosplenomegaly	+	+	+
Hematologic complications secondary to hypersplenism	+	+	+
Skeletal deterioration (bone crises/fractures)	+	–	+
Neurodegenerative course	–	+++	++
Life expectancy	6–80+ years	2 years	20–40 years
Ethnic predilection	Ashkenazi Jew	Panethnic	Swedish (Norrbottnian)

A small number of patients with the severe forms of the disease develop central nervous system damage, delayed sexual maturation, severe wasting, and eventually die. It should be noted that clinical presentations can be misleading because some of these patients can still develop underlying skeletal complications. Bone density and decreases in bone stability have been observed in 75% to 90% of patients diagnosed with Gaucher's Disease.⁶ It is important, therefore, to perform a baseline skeletal evaluation even in patients with a mild case of the disease. The femoral head is the most common initial bone site to be affected by the disease, and this area should be evaluated by magnetic resonance imaging (MRI) to assess for avascular necrosis.⁷

Bone involvement in Gaucher's disease can present as chronic bone pain or severe crises similar to those described in patients with sickle cell anemia. The severity of such episodes of bone involvement is unpredictable.

▶ **ADVANCED CONTENT**

Skeletal involvement in Gaucher's disease includes a spectrum of findings radiographically ranging from minimal bone loss and osteopenia to severe evidence of bone destruction, including osteolytic and sclerotic lesions.^{7,8} The osteolytic lesions seen on the x-ray study of the knee



FIGURE 24-2 Anteroposterior radiograph of the knee shows diffuse mottled increased density of the distal femur and proximal tibia, characteristic of widespread bone infarction in Gaucher's disease. The metaphyseal regions are broader than normal (arrow), resembling an Erlenmeyer flask deformity. (Courtesy of Charles S. Resnik, MD, Department of Diagnostic Radiology, University of Maryland Medical Center, Baltimore, MD.)

of a patient with Gaucher's disease is demonstrated in Figure 24-2.

The underlying mechanisms of the bone complications of Gaucher's disease are not well defined. It is assumed that Gaucher's cells infiltrate the medullary space and eventually replace trabecular bone and initiate a series of events that lead to osteopenia, osteolytic lesions, and osteonecrosis. Gaucher's disease is clearly a multisystemic disorder characterized not only by skeletal disease but also by organomegaly, hematologic complications, and occasionally pulmonary involvement.⁷ Patients that present with bleeding complications and nosebleeds are particularly common.

Type I Disease

Type I nonneuronopathic Gaucher's disease is the most common type of this disorder and the most common of the lipidoses. It is the most frequently inherited disorder in the Ashkenazi Jewish population. There is a remarkable degree of variability in the clinical signs and symptoms. Some patients with type I Gaucher's disease may display anemia, thrombocytopenia, massively enlarged livers and spleens, and extensive skeletal disease. In contrast, other type I Gaucher's disease patients have no symptoms at all, and the disorder is identified in their adult years only during the screening or evaluation for other diseases. The average age of onset is between 30 and 40 years. In most cases of a severe disorder, the diagnosis is made in childhood or early adulthood. Approximately two-thirds of the patients with type I Gaucher's disease are of Ashkenazi Jewish descent.⁹ The remaining one-third of patients with type I disease have a panethnic distribution.

Three clinical presentations occur in the type I nonneuronopathic form of Gaucher's disease, as shown in Table 24-2¹

Type II Disease

Type II Gaucher's disease is a much rarer form that occurs in infancy, and patients rarely survive past the age of 2 years. Type II acute neuronopathic Gaucher's disease is seen in all ethnic groups, although it is uncommon in the Jewish population. The frequency of type II disease is estimated at approximately 1 in 100,000 to 500,000.¹⁰ The hallmark of

TABLE 24-2 Clinical Presentations of Type I Gaucher's Disease	
Presentation	Findings
Mild	<ul style="list-style-type: none">• 10%–25% of patients• Asymptomatic• No need for treatment
Moderate	<ul style="list-style-type: none">• Hepatosplenomegaly• Near-normal blood counts• Normal physical appearance
Severe	<ul style="list-style-type: none">• Massive hepatosplenomegaly• Significant thrombocytopenia• Anemia, skeletal complications

type II Gaucher's disease is neurological involvement, including multiple signs such as difficulty swallowing, opisthotonos (extreme arching of the spine), and other manifestations of brainstem involvement that are noted early in infancy.¹⁰ The infant has difficulty in feeding and fails to grow. Death usually occurs before the age of 2 years. Familial intermarriage is frequently found in the infant's family history.

The clinical presentation of this type II disease is much more uniform than that observed in type I Gaucher's disease and is very severe. The disease exhibits a progressive pattern of clinical symptoms, with hepatosplenomegaly evident within the first 6 months of life and often discovered by 3 months of age. The principal cause of death in infants with type II Gaucher's disease is brainstem damage.

Type III Disease

Type III Gaucher's disease may be present from early childhood to the teenage years and is characterized by clinical and physical findings and survivals ranging between those of type I and type II.² Type III has been noted, especially in a group of children from northern Sweden, in the offspring of several related intermarriages. Neurological involvement is also characteristic of type III Gaucher's disease; however, the clinical manifestations are much more heterogeneous than those observed in type II.

Four subtypes of type III Gaucher's disease have been described, types IIIa–IIIc and the Norrbottnian variant.¹⁰

ADVANCED CONTENT

The classic form, type IIIa, usually presents itself clinically between early childhood and midadult life. Type IIIb Gaucher's disease is characterized by a clinically aggressive systemic disease with neurological involvement of isolated horizontal supranuclear gaze palsy as the major sign.¹⁰ Ocular motor disorder is the distinctive neurological feature of type IIIb Gaucher's disease. Type IIIc is a rare subtype and characterized by cardiac and aortic complications, a supranuclear gaze, hepatosplenomegaly, hydrocephalus, and skeletal abnormalities. A fourth and final subtype of Gaucher's disease type III is the Norrbottnian variant. This

rare subtype is associated with massive visceral involvement, cognitive deficits, and is historically linked to the subtype observed in northern Sweden in the 16th century.¹⁰ Generally, in type III Gaucher's disease regardless of the subtype, the more severe the neurological disease, the shorter the survival. The clinical characteristics of type IIIa and type IIIb Gaucher's disease are compared in Table 24-3.

Laboratory Testing and Results

The definitive diagnosis of Gaucher's disease is made with an assay showing the deficiency of enzyme acid β -glucocerebrosidase in the leukocytes or mononuclear cells.⁹

ADVANCED CONTENT

Rare cases of Gaucher's disease have Saposin C deficiency, which should be tested in cases where glucocerebrosidase activity is normal but the clinical picture and biomarkers point to Gaucher's disease. The diagnosis in those cases is made by sequencing for PSAP gene.¹¹

The gene for the enzyme glucocerebrosidase is located on chromosome 1q21–31. Since the characterization, cloning, and sequencing of the glucocerebrosidase gene in 1984, over 500 mutations of the *GBA1* allele have been reported.¹² The mutations include both single insertional and point mutations as well as crossover mutations, and all mutations that cause Gaucher's disease have complex effects on the properties of this enzyme. The majority of Gaucher's disease in North America and Europe involve N370S or L444P mutated alleles of the *GBA1* gene.¹³

The normal enzyme, glucocerebrosidase (acid β -glucosidase), is a lysosomal enzyme responsible for the degradation of the glucosylceramide molecule, preventing its build-up in tissue cells.³ Protein synthesis of the normal enzyme occurs in the endoplasmic reticulum, with transport to the Golgi apparatus for glycosylation and delivery to the lysosomes of the cell. Mutations at the genetic level that code for the production of this enzyme have direct effects on the catalytic activity, with decreases in enzyme from 50% to 90%.¹⁴ In addition, enzyme stability and half-life activity (normal is 60 hours) are also decreased for acid β -glucosidase as a result of these mutations.¹⁴

Peripheral blood, bone marrow, and spleen are sites most frequently examined in patients with Gaucher's disease. The peripheral blood nearly always demonstrates a moderate normocytic, normochromic anemia with thrombocytopenia because of the replacement of normal hematopoietic cells with Gaucher's cells in the bone marrow. There is pooling of blood in the enlarged spleen and some degree of ineffective erythropoiesis, with decreased incorporation of iron in erythroid precursors in the bone marrow. As a result, active signs of a compensated anemia such as polychromasia and nucleated red blood cells are usually absent on the peripheral smear. Leukocytes

TABLE 24-3 Comparison of Type IIIa and IIIb Gaucher's Disease

Characteristics	Type IIIa	Type IIIb
Clinical onset	Early childhood to midadult	Infancy to early childhood
Clinical course	Mild to moderately severe	Aggressive systemic disease
Neurological involvement	At onset; multifocal rapid jerky movements, ataxia, spasticity, dementia, and seizures	Ocular motor disorder, horizontal supranuclear gaze palsy; mild cognitive impairment

commonly decreased in number. Platelets are also usually decreased in number as a result of splenic sequestration.¹⁵

Gaucher's cells are rarely noted in the peripheral blood. Bone marrow aspirates are often the first tissue in which Gaucher's cells are detected and support the diagnosis (see Fig. 24-1). These cells are histiocytes, 20 to 100 μm in diameter, found in moderate numbers and as clumps of cells in the thickest areas of the smear. One or more round to oval nuclei are present in each cell. The cytoplasm is faintly blue with Wright's stain and has a "crumpled tissue paper" or finely folded appearance, possibly as a result of glycolipid deposition.

ADVANCED CONTENT

Electron microscopy has demonstrated that this appearance is the result of lamellar bodies stacked inside secondary phagolysosomes. These cells stain positive with periodic acid-Schiff (PAS), acid phosphatase, Sudan black B, and oil red O stains because of the accumulation of the unmetabolized glucocerebroside.

It is important to note that the presence of Gaucher's cells is not pathognomonic for Gaucher's disease because these cells may also be found in other lymphoproliferative disorders.

The spleen is variably enlarged, owing to the accumulation of masses of Gaucher's cells. The enlargement is commonly up to 10 times normal splenic weight and can cause considerable discomfort to the patient. Other organs and systems commonly affected include the liver and, in type II, the nervous system, pituitary gland, kidneys, lung, and ovaries. These organs contain massive deposits of Gaucher's cells.

The serum acid phosphatase level is increased, and isozyme measurement of this enzyme has shown that the tartrate-resistant fraction is what is increased in patients with Gaucher's disease. The common laboratory findings in Gaucher's disease are listed in Box 24-2.

Although the Gaucher's cell is associated with the disease, so-called pseudo-Gaucher's cells have also been described. They are seen in disease states with increased cellular turnover, especially chronic myelogenous leukemia, in which the phenomenon was first described. In theory, the increased cell turnover presents excess glycosylceramide to the reticuloendothelial system. The enzyme system is overwhelmed and cannot adequately metabolize all of the material. The excess is therefore

stored in histiocytes, with their end morphological expression identical to that of true Gaucher's cells.

ADVANCED CONTENT

Pseudo-Gaucher's cells are also seen in a variety of other disorders, including acute myelocytic leukemia, chronic lymphocytic leukemia, plasma cell myeloma, aplastic anemia, idiopathic thrombocytopenic purpura, thalassemia major, and some infectious diseases.³ The presence of Gaucher-like cells in patients with these diseases has no known prognostic significance. It should be emphasized that in each of these diseases there is no deficiency of the β -glucocerebrosidase, as there is in Gaucher's disease, but rather an overtaxing of a normal system.

Prognosis

As previously stated, the length of survival in patients with Gaucher's disease is variable and depends on the type. The adult form (type I) has the longest survival, with patients surviving commonly into adulthood. In the infantile form (type II), survival beyond 2 years of age is rare. Like the clinical features, survival in the juvenile form (type III) is intermediate between the first two, and patients usually live into adolescence.

ADVANCED CONTENT

Patients with Gaucher's disease seem to have an increased incidence of Parkinson's disease. The underlying mechanism is due to deficient glucocerebrosidase activity resulting in slowdown of α -synuclein degradation with accumulation of α -synuclein in the cytoplasm, forming insoluble aggregates of Lewy bodies.¹¹

A relatively increased risk of cancer in patients with Gaucher's disease has also been reported,¹⁸ primarily because of an increased incidence of hematologic malignancy. Patients with Gaucher's disease are 15 times more likely to develop a hematologic malignancy than a healthy individual. The most frequently reported hematologic malignancies in Gaucher's disease are multiple myeloma, chronic lymphocytic leukemia, Hodgkin's disease and non-Hodgkin's lymphoma, and acute leukemia.

This increased risk of malignancy can be associated with immunological abnormalities found in patients with Gaucher's disease. These abnormalities include increased helper-suppressor (T_4/T_8) cell ratio, decreased natural killer (NK) cells, polyclonal B-cell lymphocytosis, and plasmacytosis.

BOX 24-2 Gaucher's Disease: Laboratory Findings

- Normocytic, normochromic or normocytic, hypochromic anemia
- Leukopenia
- Thrombocytopenia
- Gaucher's cells in bone marrow aspirate
- Increased serum acid phosphatase
- Positive staining of Gaucher's cells in the bone marrow with periodic acid-Schiff, acid phosphatase, Sudan black B, and oil red O stains

CRITICAL THINKING QUESTION

24-1 If anemia is present in Gaucher's disease, why aren't nucleated red blood cells or other anemic markers seen? See answers to all Critical Thinking Questions at the back of this book.

Treatment

Before the advent of the newer treatment modality of enzyme replacement therapy, Gaucher's disease was traditionally managed by supportive therapy. Total or partial splenectomy was frequently performed. In addition, transfusions, orthopedic procedures, and occasionally bone marrow transplantation were used in some patients. Although potentially curative, allogeneic bone marrow transplantation is an extremely aggressive and high-risk therapy. In 1991, a major advancement occurred in the treatment of Gaucher's disease type I. The U.S. Food and Drug Administration approved the use of enzyme replacement therapy for this disorder. Gaucher's disease is the first lysosomal storage disorder for which enzyme replacement therapy is available. Enzyme replacement therapy has successfully reversed many of the clinical complications of this disorder, including correcting blood counts and reducing the organomegaly that occurs in these patients.^{3,6,18}

The first enzyme replacement therapy utilized was a purified enzyme from human placenta. This enzyme, which is an α -glucuronidase, is manufactured by Genzyme Corporation as Ceredase and has demonstrated effectiveness by reversing the signs and symptoms of Gaucher type I, nonneuropathic disease. A recombinant form of the enzyme, which is also produced by Genzyme Corporation as Cerezyme, is genetically engineered and has the advantage of being unlimited in supply. In addition, the recombinant Cerezyme has the advantage of a very low risk of transmitting any infectious agent and has a lower rate of patients developing IgG antibodies to the glucocerebrosidase enzyme.^{6,19}

ADVANCED CONTENT

An alternative treatment approach is oral substrate reduction therapy (pharmacological chaperones), which aims to decrease production of glucosylceramide, thereby reducing the substrate levels in the cell to match the residual activity of mutant enzyme.^{6,19} Pharmacological chaperones that can pass the blood-brain barrier are currently under intense investigation to potentially ameliorate neuropathic complications in type 2 and type 3 Gaucher's disease.⁶

Niemann-Pick Disease

This inherited form of lipid storage disease was first described in 1914 by Niemann and subsequently by Pick in 1933.²⁰

Niemann-Pick disease is caused by a deficiency of the enzyme **sphingomyelinase**, with a secondary accumulation of the unmetabolized lipid sphingomyelin as well as cholesterol. Sphingomyelin is a sphingophospholipid that is a common constituent of cell membranes as well as cellular organelles. As a result, a deficiency of sphingomyelinase is a serious disorder. The general characteristics of Niemann-Pick disease are summarized in Box 24-3.

A wide variety of clinical manifestations of variable severity have been reported in patients with Niemann-Pick

BOX 24-3 General Characteristics of Niemann-Pick Disease

- Inherited lipid storage disease
- Caused by a deficiency of the enzyme sphingomyelinase
- Niemann-Pick cells (lipid-laden giant foam cells) found in bone marrow aspirate, tissues, and organs
- Increased incidence in the Jewish population
- Five types: A to E have been described
- Wide variety of clinical manifestations

disease. These include growth restriction, hepatosplenomegaly, lymphadenopathy, pigmentation, and impaired neurological functions.²¹ A large number of lipid-laden giant foam cells known as *Niemann-Pick cells* can be found in affected tissues and organs (Fig. 24-3). The detection of Niemann-Pick cells in patients with this disorder is essential for the diagnosis of this disease. There is an increased incidence of Niemann-Pick disease in the Jewish population, especially in groups with common ancestry.

Classification and Clinical Findings

Because of the very different clinical manifestations of the disease, five types, A through E, have been described.²² Only types A, B, and C are discussed here. Type E, which is very rare, has been found only in adults and is characterized by a mild chronic course and a lack of neurological manifestations. Types A, B, and C of Niemann-Pick disease are compared in Table 24-4.

Type A Disease

Type A Niemann-Pick disease is also known as *infantile or classic Niemann-Pick disease*. It is the most common form, accounting for up to 85% of all cases of Niemann-Pick disease. The onset is early in infancy and is associated with failure to thrive, difficulty feeding, and restricted physical and mental development. The skin has a waxy consistency. There is often jaundice at birth and, usually, hepatosplenomegaly with a distended abdomen. The lymph nodes are enlarged as well. A cherry-red spot in the macula of the eye is found in

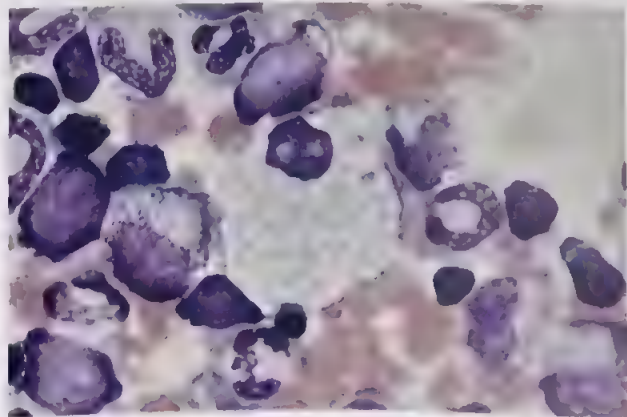


FIGURE 24-3 Niemann-Pick cell, bone marrow aspirate

TABLE 24-4 Comparison of Types A, B, and C Niemann–Pick Disease

Description	Classification		
	Type A, Infantile or Classic	Type B, Chronic or Adult	Type C, Two Forms: Infantile or Juvenile Form and Adolescent or Adult Form
Incidence	Accounts for 85% of all cases	Rare	Rare
Clinical manifestations	Jaundice at birth, hepatosplenomegaly, enlarged lymph nodes, cherry-red spot in macula of the eye, neurological symptoms, restricted physical and mental development	Hepatosplenomegaly	Hepatosplenomegaly in infantile form, splenomegaly in the adult form, neurological abnormalities, neuro-ophthalmological findings, seizures
Survival	1–2 years	Childhood or early adolescence	Juvenile or adulthood

approximately 50% of the affected infants. The neurological symptoms are more pronounced in this type of Niemann–Pick disease than in any of the other types. Deterioration is rapid, and survival past the age of 1 or 2 years is rare.

Type B Disease

Also called the chronic or adult form, type B Niemann–Pick disease is much rarer than type A, with approximately 20 reported cases in the literature. Clinical onset consisting of hepatosplenomegaly usually occurs in infancy, but the central nervous system is not involved. Individuals with this type of disease may live longer than those with type A, but they do not survive beyond childhood or early adolescence.

Type C Disease

Type C Niemann–Pick disease has been described in two forms, an infantile or juvenile form with a prolonged life span and an adolescent or adulthood form, which is generally slower in evolution and progression.²³

ADVANCED CONTENT

The primary defect in type C Niemann–Pick disease is not due to enzyme sphingomyelinase deficiency as in type A and B but rather due to mutations in *NPC1* or *NPC2* proteins.²⁴ However, this type is characterized by a milder defect in sphingomyelinase activity and an abnormality in cholesterol transport reflected in an alteration in cholesterol esterification from exogenous cholesterol.

Type C Niemann–Pick disease is an autosomal-recessive disease characterized by a gradual and ill-defined onset of neurological abnormalities, which include unsteady gait, poor motor coordination, slurred speech, dysphagia, and ophthalmoplegia (paralysis of the eye muscles).²⁵ When neurological symptoms appear, psychotic manifestations may also be predominant. Neuro-ophthalmological findings, in which the oculomotor system is often affected, are also characteristic. In addition, seizures often appear with neurological involvement, with grand mal seizures most frequently observed. Frequent seizures may contribute to the mental deterioration

observed in patients with type C Niemann–Pick disease. Hepatosplenomegaly is a constant finding in the infantile or juvenile form, whereas hepatomegaly is often absent in the adult form. Splenomegaly, however, is present in the adult form, with thrombocytopenia being a sign of hypersplenism. The characteristic foam cell, Niemann–Pick cell, sea-blue histiocytes, or a combination are a consistent finding in the bone marrow of patients with type C Niemann–Pick disease. Diagnosis of type C Niemann–Pick disease is made with the finding of the characteristic abnormality in cholesterol transport and esterification from exogenous cholesterol.

Laboratory Testing and Results

There is a distinct pattern to the histiocytes in Niemann–Pick disease. These cells are most commonly seen in bone marrow and spleen, although they accumulate throughout the body and in the nervous system (patients with type A disease). They are large cells, 20 to 90 μm in diameter, with an inconspicuous nucleus. The cytoplasm is filled with and distended by round, uniformly sized droplets of accumulated lipid, turning the cell a very pale or light blue when Wright-stained (see Fig. 24-3).

ADVANCED CONTENT

Stains producing a positive reaction with Niemann–Pick cells are the lipid stains oil red O, Sudan black, and Luxol fast blue, and acid phosphatase and nonspecific esterase. The PAS staining is weak, and the myeloperoxidase stain is negative.

The bone marrow of some adult patients with certain varieties of Niemann–Pick disease contains a mixture of Niemann–Pick cells and sea-blue histiocytes (histiocytes distended with blue-staining ceroid on Wright's stain). It is believed that the sphingomyelin is gradually metabolized to ceroid, thus generating the sea-blue histiocytes. A marrow specimen with these findings would then need to be distinguished from the entity of sea-blue histiocytosis (see the Histiocytosis section at the end of this chapter).

Other disorders that may cause Niemann–Pick–like cells in the bone marrow are GM, gangliosidosis, lactosyl ceramidosis, and Fabry's disease.

The peripheral blood is most remarkable for the vacuoles that may be found in lymphocytes and monocytes of a routine peripheral blood smear (Fig. 24-4). These vacuoles are round, and from 2 to 20 may be found within one cell. Anemia and leukopenia may be present but do not usually present any threat to the patient. Serum lipids are not usually increased. An assay of the enzyme sphingomyelinase activity in leukocytes and fibroblasts can also be performed.

Prognosis and Treatment

There may be a slightly longer survival in patients with the other types of Niemann–Pick disease, but those with type A have a very short life expectancy. Survival past the age of 2 years is uncommon. Currently there is no treatment for Niemann–Pick disease. However, successful allogeneic bone marrow transplants have been reported for type B.²⁶ In addition, research studies have focused on finding a source of enzyme replacement for sphingomyelinase²⁷ and gene therapy using retroviruses.²¹

Tay–Sachs Disease

Also known as GM₂ gangliosidosis, Tay–Sachs disease was first described in 1881 by the British ophthalmologist Warren Tay. In 1886, the New York neurologist Bernard Sachs used the term *familial amaurotic infantile idiocy* to describe this disorder. Its incidence in the Ashkenazi Jewish population is more than 250 times greater than that in the non-Jewish population. It is estimated that this high-risk group has a 1 in 25 carrier rate.²⁸ This autosomal-recessive sphingolipidosis is the result of a deficiency of the enzyme **hexosaminidase A** (HexA), with an increase of the other isoenzyme, **hexosaminidase B**. HexA enzymes are the product of two genes. HEXA and HEXB genes comprise the alpha and beta subunits of HexA. The genes are located on chromosome 15 and chromosome 5, respectively.²⁹ Inheritance of two abnormal alleles (one from each parent) accounts for almost all infantile Tay–Sachs cases in the Ashkenazi-Jewish population. The severity of the disease correlates with the level of residual enzyme activity. The unmetabolized GM₂ ganglioside accumulates in almost all tissues and has its most devastating effects within

the central nervous system and eye.²⁹ The general characteristics of Tay–Sachs disease are summarized in Box 24-4.³⁰

Clinical Findings

Although affected infants appear normal at birth, by 6 months of age both physical and mental deterioration are notable. They have an exaggerated physical response to noise (the startle reflex) beginning at age 3 to 5 months. There is a progressive loss of motor function with weakness, decreased attentiveness to surroundings, hypotonia (diminished tone of skeletal muscles), and poor head control between the ages of 6 and 10 months.³¹ In addition, a cherry-red spot in the macula of each eye is found; this is the most characteristic feature of Tay–Sachs disease. The central nervous system steadily degenerates after 1 year of age. Along with the continual deterioration, there is enlargement of the head (macrocephaly), seizures, and paralysis. Spasticity with hyperactive reflexes, deafness, and blindness follow. The neurons are greatly enlarged by accumulation of the unmetabolized ganglioside in vacuoles in the cytoplasm. In contrast to many other lipid storage diseases, the spleen, liver, and lymph nodes are not enlarged. Feeding is poor, and death occurs by four years of age. It should be noted that cherry-red spots are not pathognomonic for Tay–Sachs disease; however, this clinical finding in a Jewish infant with the absence of organomegaly is strongly suggestive of Tay–Sachs disease.³²

Laboratory Testing and Results

A deficiency of hexosaminidase A is the basic cause of this disease. Hexosaminidase A is the enzyme responsible for hydrolyzing GM₂ ganglioside, the glycolipid that accumulates in neurons. This deficiency can be demonstrated in the serum, plasma, leukocytes, and cultured fibroblasts of infants with Tay–Sachs disease.

The major site of pathology is the central nervous system, and examination of other tissues is less informative. The peripheral blood contains vacuolated lymphocytes (see Fig. 24-4). The number and size of the vacuoles are related to the duration of the disease. It is postulated, but not definitely proven, that they contain the unmetabolized lipid GM₂ ganglioside. Vacuolated lymphocytes, however, are not pathognomonic for Tay–Sachs disease because they are also seen in Niemann–Pick disease and in certain types of leukemia. Foam cells, or vacuolated histiocytes, are found in the bone marrow.



FIGURE 24-4 Tay–Sachs disease, vacuolated lymphocytes (also characteristic of Niemann–Pick disease).

BOX 24-4 General Characteristics of Tay–Sachs Disease

- Known as GM₂ gangliosidosis
- Autosomal-recessive inheritance
- Caused by deficiency of hexosaminidase A
- Higher incidence in Ashkenazi Jewish population
- Central nervous system degeneration
- Physical and mental deterioration
- Cherry-red spot in the macula of each eye
- Macrocephaly (enlargement of head)
- Seizures and paralysis
- Death by 4 years old

The presence of these cells is helpful but not diagnostic for the disease.

Because of the high frequency of this disease in certain populations, prenatal detection has taken on greater importance. Culture of fetal fibroblasts from the amniotic fluid can be undertaken to detect hexosaminidase A levels in the fetus. Mass screening programs of adults at possible risk for transmitting the disease have been undertaken, with variable success.

Prognosis and Treatment

The infantile form of Tay-Sachs disease is uniformly fatal before age 4. Enzyme replacement is now being attempted, and the results of the potential therapy are not yet known. Patients with the juvenile and adult forms of Tay-Sachs disease have longer survival than those with the infantile form, although it is quite variable. Supportive treatment remains the mainstay of this disorder, which includes management of hydration, recurrent infections, and seizures with conventional fluids, antibiotics, and drugs, respectively. Prenatal diagnosis can be performed by measuring hexosaminidase A in amniotic fluid, cultured amniocytes, or chorionic villus samples.

Mucopolysaccharidoses

The **mucopolysaccharidoses (MPSs)** are rare disorders that constitute a group of lysosomal storage diseases caused by a deficiency in one of the enzymes involved in the breakdown of mucopolysaccharides.³³

Similar to the other lipid storage disorders, the MPSs show accumulations of unmetabolized material within lysosomes (Fig. 24-5); however, it is mucopolysaccharides, not sphingolipids, that accumulate. Products are found in the reticuloendothelial system (spleen, bone marrow, liver), lymph nodes, blood vessels, brain, heart, connective tissue, and urine. The clinical severity of these disorders varies widely, with mild, intermediate, and severe forms. Multiple clinical presentations exist, including skeletal abnormalities, organomegaly, facial dysmorphism, and corneal opacities.

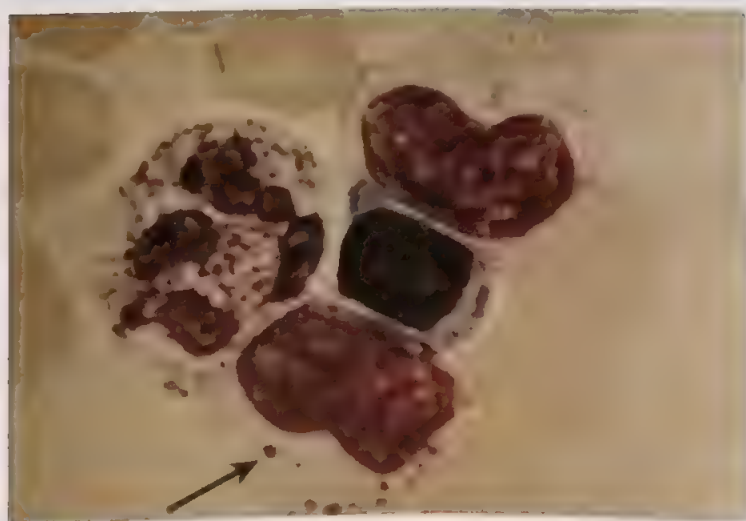


FIGURE 24-5 Hurler's anomaly. Note the Alder-Reilly bodies (arrow) that represent partially digested mucopolysaccharides which are permanent and resemble toxic granulation.

The original description of children affected with different forms of the MPSs was published within a relatively short time span at the turn of the last century. In London in 1908, Dr. John Thompson first described three young brothers with the characteristics of MPS. Gertrud Hurler elaborated on his description, describing two unrelated boys in Munich in 1918 with very similar characteristics, now known as Hurler's syndrome. In 1917, Hunter described two brothers with a constellation of abnormalities now recognized as Hunter's syndrome.

The general characteristics of MPSs are listed in Box 24-5.

Classification

The MPSs have been arranged into seven categories, but there are only four possible unmetabolized products that build up in tissues: keratan sulfate, dermatan sulfate, heparan sulfate, and chondroitin sulfate. Table 24-5 outlines an abbreviated classification scheme for MPSs.³³ With the exception of Hunter's syndrome, which is X-linked recessive, these disorders have an autosomal-recessive mode of inheritance. There does not appear to be a significant increase of affected individuals within any one ethnic group.

ADVANCED CONTENT

In terms of the biochemical classification of MPS, very different clinical phenotypes can result from different mutations at the same locus. Characterization of these mutations showed deletions for MPS I, II, and III, and a point mutation for MPS IV.³³ For example, in MPS I H (Hurler's disease) and in MPS I S (Scheie's syndrome), mutations at the same locus of the enzyme alpha (α)-iduronidase occur for both disorders, producing quite different clinical phenotypes.³³ In Hurler's disease, there is a progressive mental and physical deterioration, with death occurring usually in the first decade of life. This is in contrast to the course in Scheie's disease, in which the condition is milder; intellect is normal into adult life, and life expectancy is normal. The presence of many different mutations at the α -iduronidase enzyme locus that may be inherited in either the homozygous or heterozygous state accounts for the wide variation in clinical phenotypes. The use of molecular probes to characterize these mutations will eventually allow correlations to be made between mutations and phenotypes.

BOX 24-5 General Characteristics of Mucopolysaccharidoses

- Rare lysosomal storage disease
- Autosomal-recessive inheritance
- Deficiency of one of the enzymes involved in the breakdown of mucopolysaccharides
- Panethnicity
- Different clinical phenotypes resulting from different mutations at the same locus

TABLE 24-5 Mucopolysaccharidoses (MPSs)^{12,34}

Category	Mode of Inheritance	Accumulated Product	Enzyme Deficiency	Mutation of Gene	Clinical Features	Life Expectancy
PS I H (Hurler's)	Autosomal-recessive	*Heparan sulfate *Dermatan sulfate	α -L-iduronidase	IDUA	*Onset 6–8 months *Severe intellectual disability *Dwarfism *Large long head *Flat broad nose with upturned nostrils (coarse facies) *Corneal clouding *Hepato-splenomegaly *Valvular lesions *Coronary artery lesions *Skeletal deformities *Joint stiffness	6–10 years
MPS I S (Scheie's)	Autosomal-recessive	*Heparan sulfate *Dermatan sulfate	α -L-iduronidase	IDUA	*Onset after 5 years *Normal intelligence *Stiff joints (especially of the hands) *Near-normal height *Corneal clouding *Valvular lesions *Coronary artery lesions	Normal
MPS I H-S (Hurler-Scheie)	Autosomal-recessive	*Heparan sulfate *Dermatan sulfate	α -L-iduronidase	IDUA	*Onset infancy *Mild intellectual disability (may be normal) *Dwarfism *Facial and bony lesions of Hurler's syndrome *Cardiac lesions	Third decade
MPS II (Hunter's) (wide range of severity)	X-linked recessive	*Heparan sulfate *Dermatan sulfate	Iduronate α -sulfatase	IDS	*Mild intellectual disability to normal intelligence *Similar to Hurler's syndrome, but not corneal clouding *Retinal degeneration *Deafness *Nodular skin infiltrates	Second decade to normal
MPS III San Filippo A	Autosomal-recessive	Heparan sulfate	Heparan N-sulfatase	SGSH	Wide range of severity	
MPS III San Filippo B			α -N-acetyl-glucosaminidase	NAGLU	Wide range of severity	
MPS III San Filippo C	Autosomal-recessive	Heparan sulfate	α -glucosaminide transferase	HGSNAT	Wide range of severity	

Continued

TABLE 24-5 Mucopolysaccharidoses (MPSs)^{33,34}—cont'd

Category	Mode of Inheritance	Accumulated Product	Enzyme Deficiency	Mutation of Gene	Clinical Features	Life Expectancy
MPS III San Filippo D	Autosomal-recessive	Heparan sulfate	N-acetylglucosamine-6-sulfatase	GNS	Wide range of severity	
MPS IV A (Morquio's) (wide range of severity)	Autosomal-recessive	*Keratan sulfate *Chondroitin sulfate	N-Acetylgalactosamine, 6-sulfate sulfatase	GALNS	*Normal intelligence *Severe skeletal deformities *Dwarfism *Thoracolumbar gibbus	Third to sixth decade
MPS IV B (Morquio's)			β -Galactosidase	GLB1	*Kyphoscoliosis, facies similar to Hurler's syndrome *Corneal clouding *Valvular and coronary artery lesions *Joint hypermobility *Genu valgum	
MPS VI (Maroteaux-Lamy)	Autosomal-recessive	Dermatan sulfate	N-Acetylgalactosamine 4-sulfatase (arylsulfatase B)	ARSB	*Similar to Hurler's syndrome, but normal intelligence *Longer survival	Second decade
MPS VII (Glucuronidase deficiency disease)	Autosomal-recessive	Dermatan sulfate	β -Glucuronidase	GUSB	Variable from severe intellectual disability with dysostosis multiplex and hepatosplenomegaly to a milder form; also severe neonatal form with hydrops fetalis	Variable, 1–40 years

Source: Modified from Colmenares-Bonilla D, Colin-Gonzalez C, Gonzalez-Segoviano A, Esquivel Garcia E, Vela Huerta MM, Lopez-Gomez FG. Diagnosis of Mucopolysaccharidoses Based on History and Clinical Features: Evidence from the Bajío Region of Mexico. *Cureus*. 2018;10(11):e3617.

Clinical Findings

Many clinical abnormalities are found within each type of MPS (see Table 24-5).^{33,34} The findings in Hurler's syndrome are given in the most detail because it is considered the prototype of the MPSs.

In patients with Hurler's syndrome (MPS I), there may be a short period of apparently normal development, but this is only temporary. These individuals are abnormally short and have coarse facial features, with a broad, flat nose, widely spaced eyes, and thickened tongue and lips.³⁹ The amount of body hair is increased, dark, and especially prominent on the forehead. The skin is thickened. Patients have intellectual disability. Clouding of the corneas of the eyes is present. These individuals may have hearing loss or be completely

deaf. The heart is damaged, owing to the accumulation of mucopolysaccharides in the valves and blood vessels. There is a hump on the back and a prominent abdomen, with enlarged liver and spleen. The arms and legs are abnormal with contractures of many joints. In addition, the hands are very wide and the fingers shortened.

In Hunter's syndrome (MPS II), the changes are similar although not as severe. Corneal clouding is much less common. Patients affected with Sanfilippo's syndrome (MPS III) have a more normal stature but unfortunately many have severe neurological problems and decreased survival. Compared with patients with Hurler's syndrome, those with Scheie's syndrome (MPS I S) have more prominent corneal clouding but less abnormality in stature, facial appearance

and mental development. Patients with Maroteaux-Lamy syndrome (MPS VI) have growth and skeletal abnormalities but no intellectual disability. In Morquio's syndrome (MPS IV), patients have numerous skeletal changes, giving a markedly abnormal physical appearance; however, there is no intellectual disability.³⁴

Laboratory Testing and Results

An accurate enzymatic diagnosis should be established for all suspected cases of MPS, as clinical diagnosis alone is often impossible because of overlapping phenotypes. The diagnosis of MPS can be made by performing simple enzyme assays using leukocytes, serum, or fibroblasts.³⁵ The identification of heterozygotes, however, is still a difficult process because of the overlays of normal and heterozygous levels of enzyme activity. Molecular studies such as the cloning of complementary deoxyribonucleic acids (cDNAs) can complement accurate enzyme assays.³⁵ Several specialized substrates used for the diagnosis of MPS are now available commercially.

In contrast to findings in the other lysosomal storage diseases, nonmetabolized products may be detected in the urine of patients with MPS. The toluidine blue spot test or the turbidity test to detect acid mucopolysaccharides is the initial screening test. The spot test may be unreliable, however, with up to 32% false-negative test results in patients with Hurler's syndrome reported. Also, of note, false-positive results may be seen in the urine of normal healthy newborn infants, a phenomenon that disappears by 2 weeks of age. Any positive screening test result can be confirmed with mass spectrophotometry or by lysosomal enzyme assays.³⁶

An interesting but somewhat inconsistent finding in the peripheral blood of patients with MPS is the presence of large granules in the cytoplasm of neutrophils, lymphocytes, and monocytes. These are known as *Alder-Reilly bodies* (see Fig. 24-5) and result from an accumulation of partially digested mucopolysaccharides in the cytoplasm of cells. In polymorphonuclear leukocytes, this needs to be distinguished from toxic granulation, but the large size of the granules in MPS usually leaves little doubt. A metachromatic stain, such as toluidine blue, aids in confirmation. These granules are found with much greater regularity in bone marrow histiocytes and lymphocytes.

Prognosis and Treatment

The prognosis of the MPSs varies somewhat with the type. Patients with Hurler's syndrome may live only one decade, whereas those affected with Hunter's syndrome may live into their 20s.³³ The theoretical aid of enzyme replacement therapy has yet to be translated into practical results.

Hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy have been used successfully to treat most forms of MPS (I, II, IV, and VII).³⁶ HSCT has been proven to be a permanent treatment in many cases. Research is ongoing using substrate reduction therapy and gene therapy to treat various forms of MPS. Prenatal diagnosis is still important, including first trimester diagnosis by chorionic

villus sampling. Early amniocentesis for more sensitive lysosomal enzyme assays, use of DNA analysis for detecting mutations, and the possibility of preimplantation diagnosis of early embryos after in vitro fertilization.

CRITICAL THINKING QUESTION

24-2 Why do you think leukocytes observed on a peripheral smear contain large granules in the various lipid storage diseases?

Histiocytosis

Histiocytic disorders are a group of diseases that occur when there is an overproduction of white blood cells known as histiocytes, which can lead to organ damage and tumor formation. This group of histiocytic disorders is made up of a wide variety of conditions that can affect both children and adults. In 1987, the Histiocyte Society classified these disorders into three groups based on the types of histiocyte cells involved:

- Dendritic cell disorders
- Macrophage cell disorders
- Malignant cell disorders

Sea-Blue Histiocyte Syndrome

Although initially described in isolated case reports of young adults with an enlarged spleen, the syndrome of the sea-blue histiocyte is a genetic disorder with a benign course. The striking blue color of the histiocytes after staining with Wright's stain gives the syndrome its name.

Sea-blue histiocytes may be associated with a rare genetic syndrome or secondary to another hematologic or lipid-associated disease. Most patients receive the diagnosis before they reach 40 years of age. An early diagnosis is often an indicator of a more severe disease. Major findings on physical examination are splenomegaly and often hepatomegaly. Also described, but occurring less consistently, are abnormalities of the eyes, skin, and nervous system. Involvement of the lungs may be noted on radiographic examination while involvement of the lymph nodes is not seen.

Significant laboratory findings are usually confined to the blood. In the peripheral blood, thrombocytopenia is found with great frequency. Consequently, clinical manifestations such as epistaxis, gastrointestinal tract bleeding, and purpura may be expected. However, there is no correlation of the degree of thrombocytopenia with the size of the spleen. Blood lipid levels are normal. Abnormal liver function study results are only rarely seen.

The bone marrow aspirate is usually the site of diagnosis. Histiocytes of variable size (20 to 60 μm) are present in greatly increased numbers that contain the blue-to-green staining granules that vary in size, shape, and ability to take up the stain. Thus, not all cells will have the same staining intensity. The color of sea blue histiocytes is attributed to a high

TABLE 24-6 Characteristics of Langerhans Cell Histiocytosis

Disease	Age at Onset	Main Site(s) of Involvement	Course of Disease
Eosinophilic granuloma	Children and young adults, especially males, often no symptoms until bone fracture	Unifocal—skull, rib, femur most common	Rare spontaneous healing; most require surgical removal; occasionally, patients develop recurrence later
Hand-Schüller-Christian disease	Usually <5 years old; occasional young adult	Multifocal—bones, skin, lymphoid tissue; triad of pituitary, eye, and skull involvement is characteristic but uncommon	Spontaneous recovery in localized forms; multiple lesions require chemotherapy
Letterer-Siwe disease	Usually <3 years old	Generalized—skin, lymphoid tissue, bones, +/- bone marrow; more severe and extensive than Hand-Schüller-Christian disease	Chemotherapy has improved prognosis, which was previously considered poor

rate of intramedullary cell death causing increased deposits of phospholipids in macrophages.³⁷

Most patients with this syndrome do well and have normal life spans. Splenectomy is not always required; many patients never have the spleen removed. As previously mentioned, manifestations of the disease at an early age may imply more severe symptoms. The general characteristics of sea-blue histiocytosis include:

- Autosomal-recessive, benign genetic disorder
- Striking blue histiocytes with Wright's stain
- Splenomegaly and hepatomegaly
- Thrombocytopenia

Langerhans Cell Histiocytosis

Previously referred to as eosinophilic granuloma, Hand-Schüller-Christian Disease, and/or Letterer-Siwe Disease, these disorders were later given a common diagnosis of **Histiocytosis X**, given the cell of origin was still unknown. The advent of the electron microscope enabled scientists to

reclassify the cells as epidermal **Langerhans cells**.³⁸ Langerhans' cells are large but inconspicuous cells in the skin whose function is to process and present antigen to other cells in the area, including lymphocytes. These cells, as well as histiocytes, are normally found in small numbers in the skin and reticuloendothelial system. Epidermal Langerhans cells in histiocytosis exhibit aberrant function and differentiation with abnormal proliferation resulting in granulomatous lesions. Langerhans cell histiocytosis is a clonal disorder where more than 50% of the malignant cells exhibit *BRAF-V600E* mutations.^{38,39} Characteristics of these disorders are included in Table 24-6. Most patients with these disorders are either children or young adults. The lesions observed with these disorders are composed of inflammatory cells and epidermal Langerhans histiocytes often infiltrating bone, skin, lungs and pituitary and thus can present as single or multifocal bone lesions, may involve single system or multisystems, and may encompass the central nervous system.³⁹ Outcomes are often contingent on the extent of the disease.

SUMMARY CHART

- Lipid storage diseases range from essentially asymptomatic to severe and incapacitating, resulting in death.
- Gaucher's disease results from a deficiency of the enzyme β -glucocerebrosidase, which leads to an accumulation of unmetabolized substrate glucocerebroside in cells, predominantly the monocyte-macrophage system. This accumulation of glucocerebrosides produces the distinctive Gaucher's cells.
- Gaucher's disease has three clinically recognizable types: the adult or nonneuronopathic form (type I); the infantile, acute, or malignant neuronopathic form (type II); and the juvenile or subacute neuronopathic form (type III).
- Type I (chronic nonneuronopathic) adult Gaucher's disease is the most common type of Gaucher's disease. It occurs frequently as an inherited disorder in the

- Ashkenazi Jewish population. Clinical features include anemia, thrombocytopenia, massively enlarged liver and spleen, and extensive skeletal disease.
- Type II (acute or malignant neuronopathic) infantile Gaucher's disease occurs in infancy. Patients rarely survive past the age of 2 years. It is found in all ethnic groups, although it is uncommon in the Jewish population. The clinical presentation of type II disease is much more uniform than that of type I and is very severe. Features include difficulty swallowing, opisthotonos, and other manifestations of brainstem involvement, which are noted in early infancy.
- Type III (subacute neuronopathic) juvenile Gaucher's disease may be present from early childhood to the teenage years. Two distinct subtypes of type III have

SUMMARY CHART—cont'd

been described. Type IIIa usually presents clinically between early childhood and midadult life. Type IIIb is a clinically aggressive systemic disease, with neurological involvement characterized by isolated horizontal supranuclear gaze palsy.

- Gaucher's disease is the first lysosomal storage disorder for which enzyme replacement therapy is available.
- Niemann–Pick disease is caused by a deficiency of the enzyme sphingomyelinase, with a secondary accumulation of the unmetabolized lipid sphingomyelin as well as cholesterol. Type A is also known as infantile or classic Niemann–Pick disease. Type B is also called the chronic or adult form. Type C has been described in two forms: infantile and juvenile.

- Tay–Sachs disease, also known as GM₂ gangliosidosis, is an autosomal-recessive sphingolipidosis that occurs as a result of deficiency of the enzyme hexosaminidase A, with an increase in hexosaminidase B.
- Mucopolysaccharidoses are rare disorders that constitute a group of lysosomal storage diseases caused by a deficiency in one of the enzymes involved in the breakdown of mucopolysaccharides.
- Sea-blue histiocyte syndrome is a genetic disorder with a benign course. The striking blue color of the histiocytes with Wright's stain gives the syndrome its name.
- Histiocytic disorders represent an abnormal proliferation and accumulation of mature histiocytes, or Langerhans' cells.

CASE STUDY 24-1

A 32-year-old man visited his physician complaining of pain in his forearms and fatigue. Physical examination revealed an enlarged spleen and multiple bruises down the patient's forearms. His physician ordered the following laboratory workup:

BC count	$3.1 \times 10^{11}/L$
WBC count	$4.9 \times 10^9/L$
Hemoglobin	11.0 g/dL
Hematocrit	32%
MCV	12 fL
MCHC	3 g/dL
Platelets	$90 \times 10^9/L$
Reticulocytes	0.4%

	Differential
Segmented neutrophils	51%
Lymphocytes	41%
Monocytes	4%
Bands	1%

A bone marrow aspiration was performed at the left posterior iliac crest and revealed a histiocytic-appearing cell (see Fig. 24-2). These cells stained positive with PAS, Sudan black B, and Prussian blue.

QUESTIONS

1. This case history is representative of what lipid storage disease?
2. What further testing must be done to confirm the diagnosis?

3. Is "effective erythropoiesis" apparent in this case? Why or why not?
4. Classify the anemia according to the red blood cell (RBC) indices.
5. What treatment is available to this patient?

ANSWERS

1. The pain in the patient's forearm likely is indicative of skeletal complications seen in Gaucher's disease. The diagnosis is confirmed by the presence of an enlarged spleen, petechiae due to thrombocytopenia, the presence of anemia, which causes fatigue, and Gaucher's cells in the bone marrow, as indicated by the cells positive for PAS, Sudan black B, and Prussian blue. Additionally, the diagnosis is being made later in life for this individual and there is no neurological involvement with the disease, further indicating Type I Gaucher's disease.
2. This patient requires testing for β -glucocerebrosidase deficiency.
3. The RBC count is low, as well as the Hgb; however, the MCV and MCHC indicate that the anemia is normocytic/normochromic. This is indicative of uncompensated anemia, which is common in Gaucher's disease. Also indicative is the lack of nRBCs in the peripheral smear.
4. Normocytic, normochromic anemia.
5. The use of enzyme replacement therapy is an approved and safe treatment for this disorder.

REVIEW QUESTIONS

1. Gaucher's and Tay-Sach's disease are associated with which ethnic group?
 - a. African Americans
 - b. Ashkenazi Jews
 - c. Pacific Islanders
 - d. Alaska Natives
2. What is the enzyme deficiency seen in Gaucher's disease?
 - a. Sphingomyelinase
 - b. Hexosaminidase A
 - c. β -Glucocerebrosidase
 - d. β -Galactosidase
3. Which description best characterizes type I Gaucher's disease?
 - a. Found in any ethnic group; multiple neurological signs, including difficulty in swallowing and manifestations involving brainstem; enlargement of liver and spleen
 - b. Found primarily in Ashkenazi Jews; enlargement of liver and spleen; anemia thrombocytopenia
 - c. Found in northern Sweden; neurological disorders, bone disorders, skin pigment changes
 - d. Found in Mediterranean populations; hypermetabolic manifestations; fever, lethargy, poor musculature, bone deformities
4. Which of the following is true about Type I Gaucher's disease?
 - a. Average age onset is between 20 to 30 years old.
 - b. 75% to 90% of patients experience decreases in bone stability.
 - c. There is hallmark neurological involvement.
 - d. The disease includes ocular motor disorder.
5. What are the characteristics of Gaucher's cells?
 - a. Atypical lymphocytes with foamy cytoplasm
 - b. Hypersegmented neutrophils with Auer's rods
 - c. Large, multilobed monocytes with prominent red granules
 - d. Histiocytes with cytoplasm resembling "crumpled tissue paper"
6. What is the enzyme deficiency seen in Niemann-Pick disease?
 - a. Sphingomyelinase
 - b. Hexosaminidase A
 - c. β -Glucocerebrosidase
 - d. β -Galactosidase
7. What are the characteristics of Niemann-Pick cells?
 - a. Atypical lymphocytes with large vacuoles
 - b. Cytoplasm filled with lipid droplets, inconspicuous nucleus
 - c. Vacuolated histiocytes or foam cells
 - d. Lymphocytes with Alder-Reilly bodies
8. Which statement is true concerning lymphocytes and monocytes in the peripheral smear with Niemann-Pick disease?
 - a. Lymphocytes and monocytes are observed in decreased concentrations.
 - b. Lymphocytes and monocytes contain vacuoles.
 - c. Lymphocytes and monocytes appear activated.
 - d. There are vast increases in lymphocyte and monocyte numbers.
9. What is the enzyme deficiency seen in Tay-Sachs disease?
 - a. Sphingomyelinase
 - b. Hexosaminidase A
 - c. β -Glucocerebrosidase
 - d. β -Galactosidase
10. What are the clinical features of Tay-Sachs disease?
 - a. Waxy, jaundiced skin; restricted physical and mental development; cherry-red spot in macula of eye
 - b. Startle reflex; blindness; macrocephaly; no enlargement of liver, spleen, or lymph nodes
 - c. Abnormal facial features; deafness; increased body hair, intellectual disability; heart damage; structural deformities
 - d. Splenomegaly; hepatomegaly; eye, skin, nervous system, and lung abnormalities
11. Why does Tay Sachs have a higher effect on the central nervous system than other lipid storage disorders?
 - a. The mutation for Tay Sachs is isolated to expression in the brain.
 - b. Gangliosides are more numerous in the brain than glucocerebrosides
 - c. Lipids have little effect on the CNS and the brain.
 - d. Glucocerebrosides have a larger effect on the brain than gangliosides
12. Which cell is found in Tay-Sachs disease, but is not considered diagnostic?
 - a. Atypical lymphocytes with large vacuoles
 - b. Cytoplasm filled with lipid droplets; inconspicuous nucleus
 - c. Vacuolated histiocytes or foam cells
 - d. Lymphocytes with Alder-Reilly bodies
13. Which of the following describes a clinical feature of MPS?
 - a. Abnormally tall individuals
 - b. Closely spaced eyes
 - c. Increased, dark body hair, especially on the forehead
 - d. Thin skin

REVIEW QUESTIONS—cont'd

14. Which cell is commonly found in MPS disorders?
 - a. Large, foamy histiocytes with blue or green granules
 - b. Neutrophils with toxic granulation
 - c. Neutrophils with Döhle bodies
 - d. Leukocytes with Alder-Reilly bodies
15. Mucopolysaccharides can be detected by which test listed below?
 - a. Serum osmolality
 - b. Toluidine blue spot test
 - c. LAP score
 - d. PAS test
16. Diagnosis of sea-blue histiocyte syndrome requires which of the following analyses?
 - a. Liver lipid panel revealing increased lipids
 - b. Serum lipid panel revealing increased lipids
 - c. Peripheral smear revealing thrombocytopenia
 - d. Bone marrow analysis revealing histiocytes with blue-to-green granules
17. Which of the following must be present to diagnose Gaucher's disease?
 - a. Bone marrow examination with identification of Gaucher's cells
 - b. Assay to detect the deficiency of β -glucocerebrosidase in leukocytes
 - c. Radiological findings of bone pathology
 - d. Gene sequencing to detect the genetic abnormality

See answers at the back of this book.

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HEMOSTASIS AND INTRODUCTION TO THROMBOSIS

CHAPTER 25

Hemostasis

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CHAPTER OUTLINE

Platelets and the Hemostatic Mechanisms

Stages of Hemostasis
Vascular System

Primary Hemostasis

Platelet Structure
Platelet Function and Platelet Plug Formation

Secondary Hemostasis: Fibrin-Forming (Coagulation) System

Classification of Coagulation Factors by Hemostatic Function
Classification of Coagulation Factors by Physical Properties

Blood Coagulation: The "Cascade" Theory

Extrinsic Pathway (Factor VII)
Intrinsic Pathway (Factors XII, XI, IX, and VIII)
Common Pathway (Factors X, V, II, and I)

Thrombin-Mediated Reactions in Hemostasis

Thrombin-Mediated Platelet Aggregation
Thrombin Formation: Role of Extrinsic Pathway
Thrombin Formation: Role of Common Pathway

Thrombin-Mediated Anticoagulant Activity

Thrombin-Mediated Tissue Repair

Fibrin-Lysing (Fibrinolytic) System

Kinin System

Complement System

Laboratory Evaluation of Hemostasis

Summary Chart

Case Study 25-1

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 25-1 List the major and minor systems involved in maintaining hemostasis.
- 25-2 Compare the end products of primary and secondary hemostasis
- 25-3 Describe platelets in terms of morphology, normal concentration, and their role in hemostasis.
- 25-4 Assess the three structural zones of a platelet and correlate each with its role in adhesion, aggregation, and secretion.
- 25-5 Evaluate the steps platelets take in the formation of the platelet plug.
- 25-6 Compare bleeding issues seen in deficiencies and dysfunction of secondary hemostasis with those seen in primary hemostasis

- 25-7 Identify the organ responsible for producing coagulation factors.
- 25-8 Categorize coagulation factors into either the contact protein group, prothrombin group, or fibrinogen group.
- 25-9 Describe the initiator and end products of the intrinsic, extrinsic, and common pathways.
- 25-10 Identify the important roles of thrombin.
- 25-11 Analyze the major components in the fibrinolytic system.
- 25-12 List the components involved in the kinin system.
- 25-13 Correlate PT and APTT with their appropriate hemostatic pathways.
- 25-14 Describe the value of the thrombin time analysis.

Hemostasis is the complex process by which the body spontaneously stops bleeding and maintains blood in the fluid state within the vascular compartment. The major role of the hemostatic system is to maintain a complete balance of the body's tendency toward clotting and bleeding (Fig. 25-1).

The focus of this chapter is to provide a comprehensive review of present-day knowledge of the biochemistry, function, interaction, and regulation of each of the major components of hemostasis. Subsequent chapters further define the genetic and molecular mechanism, laboratory diagnosis, and

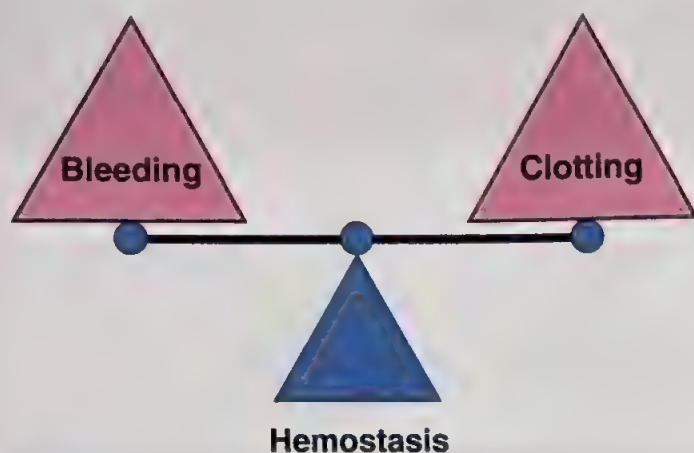


FIGURE 25-1 Hemostasis: A system in balance.

current treatment of the various thrombotic and hemorrhagic disorders of hemostasis.

Platelets and the Hemostatic Mechanisms

Normal hemostasis is both rapid and localized. Hemostasis is achieved by the highly integrated and regulated interaction of the following major systems:

- Vascular system
- Platelets
- Coagulation system
- Fibrinolytic system
- Serine protease inhibitors

Through a complex series of molecular interactions involving cells and biochemicals, a balance between procoagulant and fibrinolytic activity is achieved. A fine line separates clot formation in circulating blood from bleeding. It has long been recognized that an imbalance, either acquired or inherited, in any one of the coagulation factors can contribute to hemorrhage or thrombosis. Significant changes in blood flow have a profound effect on cellular function, particularly endothelial cell function. Mechanical or pathological disruption of intact endothelium within the vessel wall initiates localized hemostatic and thrombotic responses. If the vascular injury exceeds the capacity of the platelets mediated by thrombin to form a hemostatic platelet plug, then the clinical signs and symptoms of hemorrhage occur. Stagnation of blood flow from arterial disease or mechanical impedance disturbs the endothelial cell anticoagulant effect, thus leading to the formation of a thrombus (clot) and hypercoagulable states.¹

Stages of Hemostasis

Hemostasis can be divided into two stages: primary and secondary. **Primary hemostasis** refers to the vascular injury response that produces a platelet plug at the site of damage. Primary hemostasis serves to limit bleeding immediately through the formation of a loose platelet plug by adhering to the endothelial wall at the site of injury. Platelets then release potent coagulant compounds that enhances their aggregation, forming a more substantial plug. These platelets then provide

a phospholipid surface for activated coagulation enzyme complexes.² The interaction of platelets and vascular endothelium is the first step in a series of reactions that lead to thrombus formation and serve to halt bleeding after vascular injury.^{2,3} However, the primary response alone is ineffective for any duration of time.

Secondary hemostasis involves the enzymatic activation of the coagulation proteins to produce fibrin from fibrinogen. Soluble fibrin monomers polymerize and are then cross-linked into insoluble strands that serve to stabilize the loose platelet plug formed in *primary hemostasis*. Defects in secondary hemostasis decrease fibrin production and reduce the stability of the formed clot.²⁻⁴ The cascade of coagulation reactions is a result of complexes that form among enzymes, substrates, phospholipid surfaces, and cofactors.

The stable clot is managed and gradually dissolved through a process known as *fibrinolysis*. **Fibrinolysis** involves the proteolytic digestion of fibrinogen and fibrin by the enzyme plasmin. Through the release of platelet-derived growth factor (PDGF), vascular healing and repair is promoted, completing the process of hemostasis.²

In general, the relative importance of the hemostatic mechanisms varies with the vessel size; the larger the vessel, the more hemostatic system involvement is required to seal the breach (Table 25-1). Basically, the larger the area of bleeding, the larger the vessel involved. Ecchymoses, epistaxis, petechiae, gastrointestinal, and genitourinary bleeding represent mucocutaneous hemorrhage highly suggestive of qualitative or quantitative platelet disorders.³ Common sources and types of bleeding are listed in Table 25-2.

Vascular System

The blood vessel, with its smooth and continuous endothelial lining and fibrous coat, is designed to facilitate blood flow as well as participate in the process of hemostasis. The structure of the vessel wall, which includes the outermost layer (tunica

TABLE 25-1 Vessels and General Breach-Sealing Requirements

Vessel	Relative Sizes	General Breach-Sealing Requirements*
Capillary	Smallest	Generally direct sealing
Venule	↓	Mostly fused platelets
Arteriole		Mostly fused platelets
Vein		Vascular contraction, fused platelets, perivascular and intravascular hemostatic factor activation
Artery	Largest	Great vascular contraction, more fused platelets, greater perivascular and intravascular hemostatic factor activation

*In general, the larger the vessel, the more hemostatic system involvement is required to seal the breach.

TABLE 25-2 Common Sources and Types of Bleeding

Source	Type
Capillary, arteriole, venule	Pinpoint petechial hemorrhage (diapedesis or leakage of blood out of small vessels)
Veins	Ecchymosis (large, ill-defined soft tissue bleeding)
Artery	Rapidly expanding "blowout" hemorrhage

adventitia), the middle layer (tunica media), and the inner layer (tunica intima), is outlined in Table 25-3.

Maintaining the integrity of the vessel wall is a function of the endothelial cells, the connective tissue of the subendothelium, and platelets. A primary feature of normal intact endothelium is that it is *nonthrombogenic*, that is, it is resistant to platelets, leukocytes, and coagulation proteins. Platelet and endothelial cell interaction is modulated by the prostaglandin PGI_2 (prostacyclin).⁵ PGI_2 is synthesized by endothelial cells from arachidonic acid (a membrane phospholipid) and has an antagonistic effect on platelet adhesion and platelet aggregation, thus serving to locally limit the hemostatic response. Release of t-PA by the damaged endothelial cell provides the mechanism for clot dissolution, necessary to reestablish vascular patency.⁶ Tissue factor pathway inhibitors (TFPIs) control activation of a portion of secondary hemostasis and inhibit coagulation factors VII/TF and Xa. Heparan sulfate (endogenous form of the pharmaceutical drug heparin) enhances the activation of antithrombin, which works to inhibit thrombin. Thrombomodulin serves as a cofactor in the activation of protein C, which works to inhibit thrombin by inactivating coagulation factors V and VIII.

Once the vessel wall integrity is disrupted, the vascular system acts to prevent bleeding and promote coagulation. Endothelial disruption, such as a cut in the blood vessel, provides binding sites for collagen and von Willebrand factor (vWF). VWF binds to collagen, a structural protein, and supports platelet adhesion. VWF can bind to platelets through the glycoprotein (GP) Ib/IX/V complex and fibrinogen, as

well as fibronectin through integrin.⁷ These "sticky" proteins are thought to participate in the formation of a bridge from platelets to subendothelial connective tissue. Figure 25-2 shows a scanning electron micrograph (SEM) demonstrating platelet adherence at the site of endothelial loss compared with the normal smooth contour of the endothelial cell. Platelets are incorporated into the vessel wall, releasing a substance called platelet-derived growth factor (PDGF) that nurtures the endothelial cells, maintaining normal vascular integrity and thromboresistant properties.⁵

In addition, platelet secretion of a prostaglandin, thromboxane A_2 (TXA_2), and serotonin further promotes vasoconstriction. Endothelial release of tissue factor (formerly known as tissue thromboplastin) during vascular injury represents one of the body's major procoagulant abilities.^{8,9} The vascular response involved in the hemostatic mechanisms usually lasts less than 1 minute. The major substances contained within the endothelium, subendothelium, or both that play primary roles in hemostasis are listed in Table 25-4.

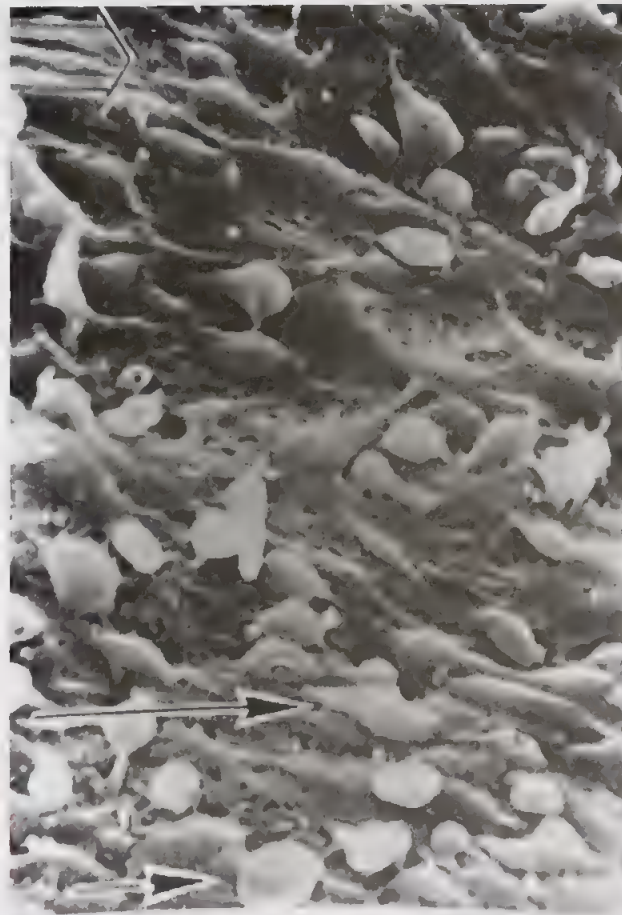


FIGURE 25-2 SEM of platelet adherence at the site of endothelial loss. Short arrow points to a discoid intact platelet with a single pseudopod; long arrow points to an elongated adherent platelet; top double arrow marks densely adherent platelets appearing as elongated humps fused to the subendothelial layer. (From Cotran, E. Robbins Pathological Basis of Disease. 1st ed. Philadelphia: WB Saunders; 1979, p. 120, with permission.)

TABLE 25-3 Vessel Layer Composition and Function

Vessel Layer	Composition and Function
Tunica adventitia	Connective tissue cell support
Tunica media	Elastic tissue and smooth muscle, controlling vasoconstriction and sometimes vasodilation
Tunica intima	Broad flat endothelial cells with an underlying basement membrane supported by a few connective tissue cells, providing a smooth surface but facilitating migration of cells through the spaces as needed

TABLE 25-4 Major Substances Present in the Endothelium and/or Subendothelium and Their Functions in Hemostasis

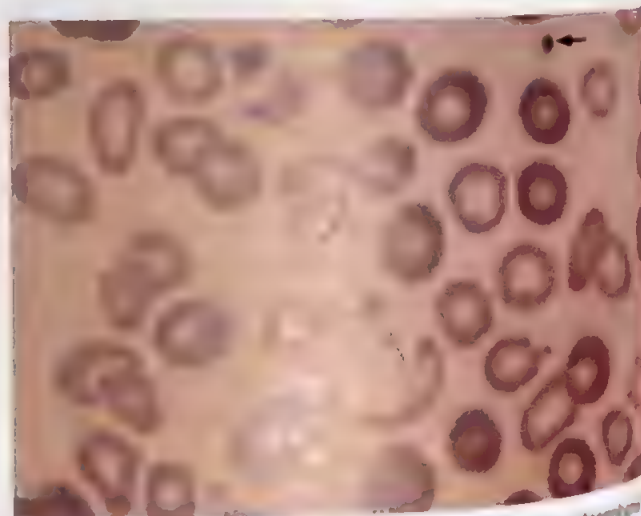
Substance(s)	Role	Function(s) in Hemostasis
Prostacyclin (PGI ₂)	Anticoagulant	Synthesized by endothelial cells and physiologically inhibits platelet aggregation and limits thrombus formation beyond the damaged vessel
Tissue plasminogen activator (t-PA)		Serine protease secreted by endothelial cells that regulates fibrinolysis, when bound to fibrin, t-PA activates plasminogen to form plasmin
Tissue factor pathway inhibitors (TFPIs)		Glycoproteins found on endothelial surfaces that serve as an anticoagulant by inhibiting factors VIIa/TF and Xa
Heparan sulfate		A glycosaminoglycan that has anticoagulant activity and contributes to the activation of antithrombin
Thrombomodulin and protein C receptor		Found on endothelial surfaces and work as a cofactor in protein C activation when bound to thrombin
Injured endothelial lining	Coagulant	Promotes vasoconstriction
Collagen		Binds vWF and platelets
von Willebrand factor (vWF)		Protein synthesized by endothelial cells and megakaryocytes; primarily binds to platelet membrane receptor GP Ib/IX/V and collagen to promote adhesion; also functions as a carrier for factor VIII, providing factor stability in circulation; may also bind to platelet GP IIb/IIIa in conditions of high shear stress to promote adhesion
Tissue factor (TF)		A lipoprotein released after vascular trauma that initiates coagulation by activating factor VII with ionized calcium (extrinsic pathway) and contributes to the activation of factors X and IX (intrinsic pathway)
Plasminogen activator inhibitors (PAIs)		Proteins found in endothelial cells that regulate fibrinolysis by neutralizing the activity of plasmin, plasminogen, and t-PA; associated with risk of thrombosis

ADVANCED CONTENT

In the absence of platelets, a large number of red blood cells migrate through the vessel wall, enter the lymphatic drainage, and appear as petechiae or purpura in the skin or mucous membranes. The process of maintenance of normal vascular integrity, involving nourishment of the endothelium by the platelet or actual incorporation of platelets into the vessel wall, utilizes a small minority of the platelets in circulation but is nevertheless an important function.

Primary Hemostasis**Platelet Structure**

Platelets are anucleated cytoplasmic fragments measuring 2 to 4 μm in diameter that originate from bone marrow megakaryocytes. On Wright's stain, platelets appear as pale blue cells with fine azurophilic granules (Fig. 25-3). During megakaryocytopoiesis, the interval from megakaryoblast to production of platelets is approximately 1 week. In the peripheral blood, about 70% of the platelets are circulating, whereas 30% are sequestered in the microvasculature of the spleen and serve as functional reserves after their release from the bone marrow. Platelets survive for 7 to 10 days in circulation and are active in hemostasis.

**FIGURE 25-3** Normal platelets: Wright-stained blood smear (peripheral blood).

The normal platelet count ranges from 150,000 to 450,000 per microliter (μL), depending on the methodology employed. Normal platelet function in vivo and in vitro requires more than 100,000 platelets per microliter.¹⁰ It is unusual to find a patient with a platelet count greater than 20,000 μL to have a major hemorrhage. Spontaneous hemorrhage may occur when platelet count falls to 10,000 or lower.¹¹ Assuming normal platelet function, a platelet count greater than 50,000 μL will minimize the chance of hemorrhage during surgery.

Platelets must be adequate in both number and function to participate optimally in hemostasis. Platelets participate in hemostasis by:

1. Providing a negatively charged phospholipid surface
2. Releasing substances that mediate vasoconstriction, platelet aggregation, coagulation (thrombin generation), and vascular repair
3. Providing surface membrane glycoproteins (GP) such as GP IIb and GP IIIa to attach to other platelets via fibrinogen, and GP Ib/IX/V to bind to collagen and subendothelium via vWF¹²

A basic understanding of the platelet's ultrastructure and its organelles is crucial to understanding how the platelet performs each of its vital functions (Fig. 25-4). Each of these individual functions can be assessed by clinical testing; however, all functions are required during the formation of the hemostatic platelet plug, known as primary hemostasis.

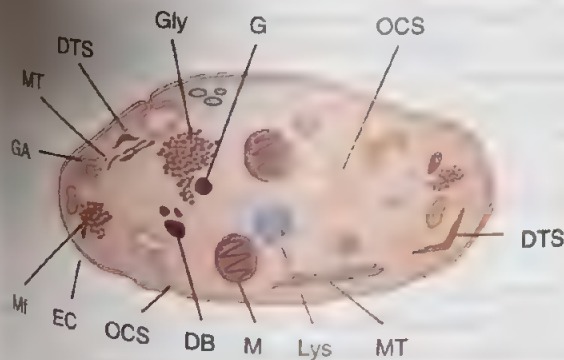
The platelet structure is quite distinct, leading to subdivision into three defined zones, each possessing unique functional capabilities. These zones are prominently delineated by the circumferential band of microtubules found in the platelet, as seen in Figure 25-5. The three described zones and their contents are listed in Box 25-1. Impaired cellular function of the platelet membrane, cytoskeleton, granular constituents, and secreted proteins often leads to platelet dysfunction and abnormal hemostasis.

Peripheral Zone

The peripheral zone is a complex region of the platelet consisting of the glycocalyx (an amorphous exterior coat), platelet



FIGURE 25-5 Internal anatomy of a stimulated platelet. Circumferential band of microtubules (MT) leads to reorganization of the internal structure of the platelet into three zones. The peripheral zone (PZ) is the region external to a circumferential band of microtubules (MT). The intermediate zone (IZ) (encircling arrows) includes the microtubules and the closely adjacent cytoplasmic material. The central zone (CZ) is internal to the microtubule band and contains many organelles such as granules (G), dense bodies (DB), dense tubular system (DTS), lysosomes (Ly), mitochondria (M), and many profiles of the open canalicular system (OCS). Magnification $\times 49,700$. (From Barnhart, MI. Platelet responses in health and disease. *Mol Cell Biochem.* 1978;22:115, with permission.)



DTS	MT	Microtubules
Gly	G	Other granules
OCS	DB	Dense bodies
DB	DTS	Dense tubular system
GA	M	Mitochondria
Mf	Gly	Glycogen lakes (particles)
EC	Lys	Lysosomes
OCS	OCS	Open canalicular system
DB	Mf	Microfilaments
M	EC	Exterior coat (glycocalyx)
Lys	GA	Golgi apparatus

FIGURE 25-4 Discoid platelets; (top) summary diagram of the platelet organelles; (bottom) transmission electron micrograph (TEM) of cross-sectioned platelets illustrating basic ultrastructure.

membrane, numerous deeply penetrating surface-connecting channels known as the open canalicular system (OCS), and a sub-membranous area of specialized microfilaments (see Fig. 25-4).

A number of glycoproteins anchored in the platelet membrane are present in the glycocalyx and mediate the critical events of platelet adhesion and aggregation. Specific binding of platelet membrane receptors to adhesive macromolecules such as fibronectin and vitronectin in plasma or collagen in vascular subendothelium further facilitates the spreading of platelets on the damaged vessel wall and leads to platelet activation and the formation of large platelet aggregates.⁷ Platelet membrane glycoproteins serve as receptors and facilitate transduction of activation signals across the platelet membrane in response to various external stimuli.

The membranous surface-connecting system referred to as the open canalicular system (OCS) consists of tubular invaginations of the plasma membrane that articulate throughout the platelet even though it is part of the peripheral zone. Chemical

BOX 25-1 Platelet Ultrastructural Zones**Peripheral Zone (stimulus receptor/transmitter region)**

- Glycocalyx
- Platelet membrane
- Open canalicular system (OCS)
- Submembranous region

Sol-Gel Zone (cytoskeletal/contractile region)

- Circumferential microtubules
- Microfilaments

Organelle Zone (metabolic/organelle region)

- Granules
 - Alpha (α) granules
 - Dense granules
 - Lysosomes
 - Glycogen granules
- Mitochondria
- Dense tubular system
- Peroxisomes

substances stored in the dense and alpha (α) granules of the platelet are released to the exterior through the OCS. The OCS also facilitates the collection of plasma procoagulants that aid in fibrin formation by providing increased surface absorptive area. This membrane system appears to be the calcium-regulating mechanism of the platelet.

ADVANCED CONTENT

The platelet membrane, similar to other plasma membranes, contains a phospholipid component. Activated platelets undergo shape change, develop stickiness, and expose platelet membrane phospholipids. Platelet factor 3 (PF3) is known to move to the outer surface of the platelet membrane, thus allowing for the assembly of the vitamin K-dependent coagulation factors involved in secondary hemostasis.¹³ PF3 serves as a cofactor in the complex. Coagulation factors V and VIII are also present on the surface of the platelet membrane, as are various platelet factors (PFs) that participate in the formation of fibrin. At least seven PFs have been identified (Box 25-2).

BOX 25-2 Platelet Factors (PF) 1 to 7

1. PF1 - Coagulation factor V
2. PF2 - Thromboplastin-like material
3. PF3 - Platelet thromboplastin*
4. PF4 - Antiheparin factor
5. PF5 - Fibrinogen coagulant factor
6. PF6 - Antifibrinolytic factor
7. PF7 - Platelet cothromboplastin

*Most important

Sol-Gel Zone

The term cytoskeleton is often used to describe the network of microtubules, microfilaments, and submembranous filaments.

Microtubules encase the entire platelet, maintaining its discoid shape. Microtubules are composed of protein subunits called *tubulin*. In the stimulated platelet (see Fig. 25-5), contraction of the circumferential band of microtubules toward the center of the platelet appears to be responsible for the movement of organelles toward the center and their reorganization, which facilitates the secretory process.²

Microfilaments are randomly interwoven throughout the cytoplasm of the platelet and are composed of two contractile proteins, actin and myosin. Also present is thrombosthenin, a contractile protein similar to actomyosin.² Actomyosin is a complexed actin and myosin. Microfilaments can change from an unorganized gelatinous state to organized parallel filaments capable of contraction within seconds as the platelet shape changes.

ADVANCED CONTENT

Microtubules disappear from the center of the platelet after secretion and reappear in other peripheral areas such as pseudopods. Microtubules appear to monitor the intense contraction of platelets, preventing platelet secretion in response to only minimal stimulation and thereby regulating the degree of platelet response to external stimuli.

Organelle Zone

The organelle region is responsible for the metabolic activities of the platelet. Like many other cells, platelets possess mitochondria and various cytoplasmic granules. Unlike most cells, platelets are anucleated and do not possess either a Golgi body or rough endoplasmic reticulum.

Generally, the most numerous organelles are the platelet granules, which are heterogeneous in size, electron density, and chemical content. Platelets contain three morphologically distinct types of storage granules: dense granules, alpha (α) granules, and lysosomes.

The α granules are most numerous (20 to 200 per platelet) and store several different substances. The content of platelet granules and their major functions in hemostasis are listed in Table 25-5.¹⁴

Dense granules, or bodies, are smaller and fewer in number (2 to 10 per platelet) and appear as dense opaque granules in transmission electron microscope (TEM) preparation. Dense granules store ADP, ATP, ionic calcium, and serotonin.

Lysosomes appear similar to the azurophilic granules found in granulocytes and contain microbicidal enzymes, neutral proteases, and acid hydrolases. Proteases may contribute to disruption of the subendothelial structure after vascular injury. Glycogen granules are also found within the organelle zone and function in platelet metabolism.

The contents of both α and dense granules are released during secretion into the OCS. Secretion promotes the recruitment of

TABLE 25-5 Chemical Contents of Platelet Granules and Their Major Functions in Hemostasis

Granules	Function
Alpha Granules: Platelet-Specific Proteins	
Beta-thromboglobulin (β -TG)	Inhibits heparin; chemotactic; promotes smooth muscle growth for vessel repair
Platelet factor 4 (PF4)	Inhibits heparin
Platelet-derived growth factor (PDGF)	Promotes smooth muscle growth; involved in atherosclerosis and lipid metabolism
Thrombospondin	Promotes platelet-to-platelet interaction; mediates cell-to-cell interaction
Factor V	Once activated complexes with Xa to convert prothrombin to thrombin
Alpha Granules: Plasma Proteins	
Fibrinogen	Fibrin formation
von Willebrand factor (vWF)	Promotes platelet adhesion
Factor V	Cofactor in fibrin formation
Factor VIII	Cofactor in fibrin formation
Fibronectin	Cellular adhesion molecule; promotes platelet spreading
Albumin	Uncertain
High molecular weight kininogen (HMWK)	Activation of the intrinsic pathway via contact
α_2 -Antiplasmin	Inhibits plasmin
Plasminogen	Precursor to plasmin; functions in fibrinolysis
Dense Granules	
ADP (nonmetabolic)	Promotes platelet aggregation
ATP (nonmetabolic)	Uncertain
Calcium	Primary and secondary messenger regulates platelet activation/aggregation
Serotonin	Promotes vasoconstriction

The dense tubular system (DTS) is another important structure present in the cytoplasm of the organelle zone of platelets. Similar to the sarcotubules in skeletal muscle, the DTS is derived from the smooth endoplasmic reticulum (ER) of immature megakaryocytes. The DTS is the site of prostaglandin and thromboxane synthesis and sequestration of calcium. It is primarily the release of calcium from the DTS that triggers platelet contraction and subsequent internal activation of platelets.

ADVANCED CONTENT

Platelet-derived growth factor (PDGF) is a mitogen stored in and secreted from the α granule of the platelet. Specific receptors for PDGF have been isolated on cultured fibroblasts and smooth muscle cells.¹⁰ When vessel injury occurs and platelets are activated, PDGF is secreted, stimulating endothelial cell migration and proliferation of smooth muscle growth, thereby mediating wound healing and vascular repair. PDGF is an important growth factor and is thought to be linked to the pathological development of atherosclerosis through its influence on lipid metabolism. PDGF also demonstrates chemotactic properties for neutrophils and monocytes, as well as fibroblasts and smooth muscle cells. The characteristics of PDGF help to limit the hemostatic response; thus, the platelet plug remains local to the site of vascular injury.

Platelet Function and Platelet Plug Formation

Numerous stimuli can trigger platelet activation, which may be transient, reversible, or irreversible. **Activation** refers to several separate responses of platelet function that include adhesion, shape change, secretion, and aggregation. Platelets respond in a graded fashion, depending on the strength and duration of the stimuli as well as the physiological or pathological state of the platelet.

In the initial stage of activation, platelets form pseudopods as they begin to contract. As activation progresses, contraction and pseudopod formation progress, organelles including the α granules and dense bodies are reorganized to the center of the platelet, and further contraction causes the granules to spill their contents into the OCS, which shunts the contents to the outside of the platelet.¹⁵ Adjacent platelets are activated through receptor contact with the granular contents, amplifying the activation process. Therefore, it may be said that platelet activation spans shape change, platelet adhesion, granular secretion, cytoskeletal reassembly, and platelet aggregation.

Before proceeding further, the reader should review the structure of the platelet to visualize and understand subsequent events that occur in the platelet at the ultrastructural level during hemostasis (see Fig. 25-4). The events that occur in primary hemostasis after injury to a vessel are outlined in Table 25-6.

Adhesion and aggregation of platelets to the site of vascular damage occur in concert with cellular activation. These cellular events are mediated through specific receptors on the

additional platelets to the platelet aggregate at the site of vascular injury. Platelet secretion is an energy-dependent reaction that relies on the metabolic function of mitochondria. The estimated 10 to 60 mitochondria present per platelet require glycogen as their source of energy for metabolism.¹¹ In the resting platelet, ATP (energy) production is generated by glycolysis and the oxidative Krebs cycle. In the activated state, about half the ATP production in platelets occurs through the glycolytic pathway.

TABLE 25-6 Events that Occur in Platelets After Vessel Injury

Event	Purpose
Vasoconstriction	Regulates blood flow in damaged vessel; increases concentration of biochemicals to promote platelet activation events
Platelet shape change	Signals intracellular activation, leading to platelet plug formation
Platelet aggregation	A platelet-to-platelet interaction mediated by fibrinogen, Ca^{2+} , and platelet membrane activated glycoprotein IIb/IIIa complex
Platelet plug	Adhesion and aggregation of platelets to the site of injury
Platelet secretion	Release of chemical constituents contained in various granules; amplifies platelet response
Stabilization	Platelet plug stabilized by formation of fibrin mesh over the platelet aggregates

platelet membrane. The membrane is the key to the interaction of extracellular agonists with intracellular biochemicals. A series of complex intracellular events occur that lead to further biochemical and morphological change of the platelet. The culmination of these processes permits the platelet to perform its vital role in hemostasis, assuming there is normal platelet number and function.

Adhesion

Damage to the endothelial monolayer exposes flowing blood to the subendothelial connective tissue matrix, which is composed of adhesive molecules (i.e., collagen, vWF, fibronectin, and thrombospondin). The specific binding of platelet receptors to these adhesive molecules in plasma or vascular subendothelium mediates **adhesion**. Adhesion is a reversible process. The principal mechanism of adhesion involves three critical components:

1. vWF, a plasma protein that links the platelet to subendothelial binding sites
2. A platelet membrane receptor complex, GP Ib/IX/V
3. Collagen fibers^{7,12}

The adhesion of platelets to collagen and vWF facilitates platelet spreading on the subendothelial surface, which promotes release of dense granules' contents, leading to platelet aggregation. Activation of additional platelets, through the interaction with either collagen or other mediators (such as thrombin, ADP, or TXA_2), amplifies the response and provides a positive feedback mechanism that recruits more platelets into the site.¹⁴

Evidence indicates that vWF is synthesized by endothelial cells and megakaryocytes. Once made, vWF is released into

the plasma where it is absorbed onto the surface of the platelet bound to its receptor, GP Ib/IX/V (Fig. 25-6) or incorporated into the subendothelium. Platelets thus adhere to the area of injury at the endothelial lining, acting to arrest the initial episode of bleeding. When injury occurs at a site of increased blood flow (e.g., arteries), the utilization of GP VI binding to collagen can be necessary to secure platelets to the site. In Figure 25-7, a TEM demonstrates platelet adhesion to subendothelial connective tissue at the focus of endothelial loss. A decrease in platelet number as well as platelet dysfunction results in increased bleeding.

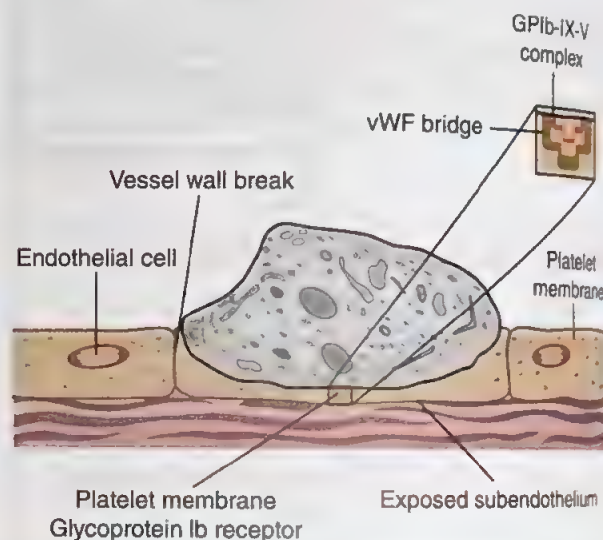


FIGURE 25-6 Pictorial representation of platelet adhesion to subendothelium through von Willebrand factor (vWF) bridge.

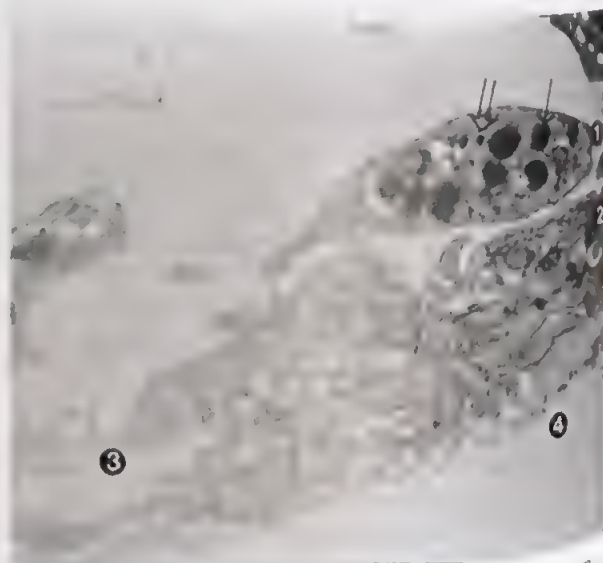


FIGURE 25-7 TEM of platelet adherence to subendothelial connective tissue at the focus of endothelial loss. (1) Intact platelet with pseudopods (thin arrow indicates α granule; thick arrow indicates dense body). (2) Partially degranulated platelet, (3) degranulated platelet "ghost," (4) internal elastic lamina. (From Cotran, E. Robbins Pathological Basis of Disease 1st ed. Philadelphia: WB Saunders; 1979, p. 116, with permission.)

ADVANCED CONTENT

GP Ib/IX/V is a leucine-rich complex composed of two molecules each of GP Ib α , GP Ib β , and GP IX, with one molecule of GP V. vWF binds directly to GP Ib α molecules, while GP Ib β interacts with actin molecules. GP IX and V help to hold the complex together.

Defects in Gp Ib/IX/V lead to **Bernard-Soulier syndrome (BSS)**, which is characterized by mucocutaneous bleeding, thrombocytopenia, and giant platelets. Deficiency in von Willebrand factor causes **von Willebrand disease (vWD)**, which is the most common autosomal inherited bleeding disorder. There is reduced or absent ristocetin-induced aggregation in both BSS and vWD. However, normal plasma containing von Willebrand factor could correct the defect in the patients with vWD, which is helpful to distinguish it from BSS.^{5,16} (See Chapter 26.)

Shape Change

The interaction of circulating platelets with external stimuli or agonists results in a series of complex reactions known collectively as **platelet activation** that precede the ultimate activation event of platelet aggregation and platelet secretion. Platelet activation occurs when an external agonist interacts with its specific membrane receptor on the platelet. Signal transduction

occurs, transmitting the signal from outside the cell to inside the cell. Activation of second messenger pathways within the platelet leads to further intracellular biochemical changes, culminating in platelet activation events such as shape change, secretion, cytoskeletal reassembly, and platelet aggregation.⁵ Following vessel injury and exposure to external stimuli, platelets change shape from circulating discs to spheres with pseudopods, indicating platelet activation (Fig. 25-8).

Many agonists such as collagen, ADP, thrombin, and TXA₂ alter the internal levels of cytosolic calcium, thus promoting shape change.⁵ The normal discoid shape of the platelet is defined by the circumferential microtubules. Increases in cytosolic calcium cause dissolution of the circumferential microtubules, thereby altering platelet shape.¹⁷ Shape change is thought to represent the most sensitive parameter of platelet activation. TXA₂ and ADP are potent platelet agonists when bound to their specific membrane receptors. During platelet activation in response to thrombin, two platelet-derived agonists (ADP and TXA₂) are released, serving to amplify intracellular events that result in platelet secretion and platelet aggregation.¹⁵ When platelets become activated, shape change and exposure of platelet membrane phospholipids such as PF3 occur. Exposure of PF3 promotes the assembly of vitamin K-dependent factors on the platelet membrane surface. Dependent on the strength of the agonist, shape change and signaling platelet activation may be followed by platelet aggregation.

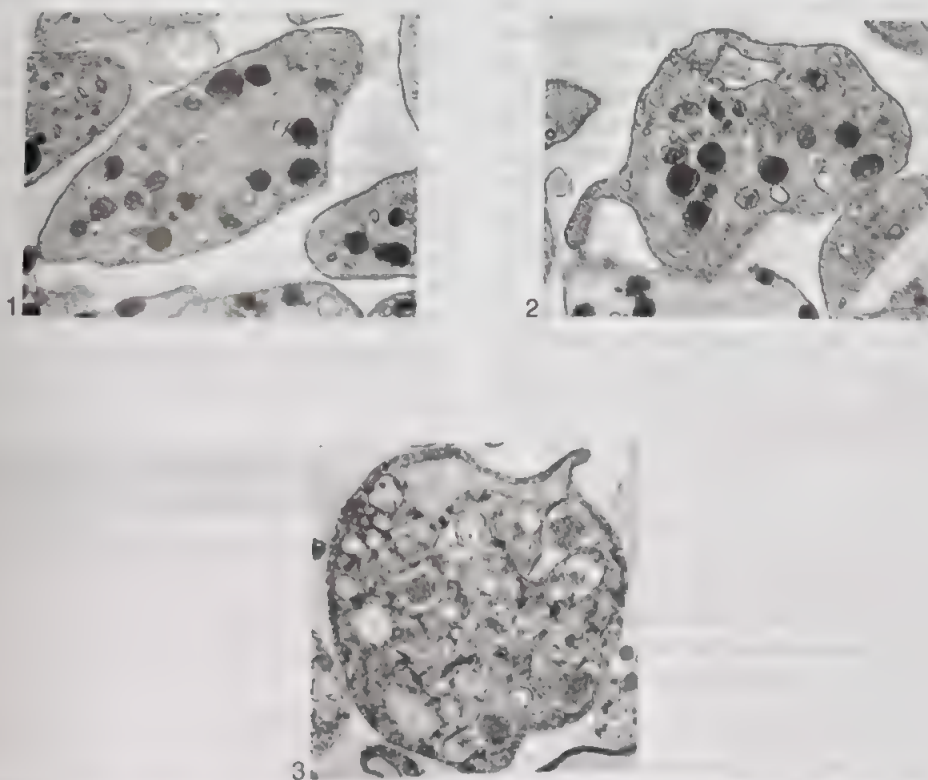


FIGURE 25-8 TEM showing disk to sphere transformation of an activated platelet. Note progression from (1) disk shape to (2) pseudopod formation to (3) degranulated ballooned sphere.

▶ ADVANCED CONTENT

ADP activates platelets through three receptors, P2Y₁, P2Y₁₂, and P2X₁. P2Y₁ and P2Y₁₂ receptors are G-protein coupled receptors and are important for platelet activation, shape change, aggregation, TXA₂ generation, and thrombus formation. Clopidogrel and ticlopidine can block P2Y₁₂ receptor and are used in the treatment of thrombotic disorders. P2X₁ receptor is an ion channel that leads to calcium influx upon activation.¹⁸

Aggregation

Platelet-to-platelet interaction is known as **aggregation** and usually begins 10 to 20 seconds after vascular injury and platelet adhesion.^{2,10} Platelet aggregation is an energy-dependent process that requires ATP, which is primarily derived from glycolysis. Ionized calcium (Ca²⁺), fibrinogen receptors GP IIb and IIIa, and fibrinogen are necessary for platelet aggregation. Platelet aggregation requires a conformational change in platelet membrane receptor GP IIb/IIIa (and GP V), thus allowing the binding of fibrinogen.^{15,19}

▶ ADVANCED CONTENT

Gp IIb/IIIa is a highly abundant platelet receptor, and it does not bind fibrinogen on non-stimulated platelets. Gp IIb/IIIa receptor can transmit information bidirectionally across the plasma membrane. Upon platelet stimulation, Gp IIb/IIIa is converted to a high-affinity fibrinogen receptor through “inside-out” signaling. This process is mediated by changes in the intracellular cytosolic portion of the receptor, resulting in the conformational changes on the external cell surface. Fibrinogen binds Gp IIb/IIIa receptors between two platelets that enforce and stabilize thrombus formation. The “outside-in” signaling occurs when Gp IIb/IIIa binds to immobilized vWF, and the cytosolic portion of Gp IIb/IIIa receptor binds to the platelet cytoskeleton, which causes platelet spreading and clot retraction. GP IIb/IIIa receptor antagonists, such as abciximab, tirofiban, and eptifibatide, are platelet-selective because the receptor is only on platelets.²⁰

The binding of the two symmetric ends of fibrinogen to different platelets promotes platelet-to-platelet interaction, thus leading to the formation of a large platelet aggregate. Calcium and/or magnesium, in the form of divalent cations, are also required for fibrinogen binding. Once fibrinogen binds to this membrane complex, extracellular Ca²⁺-dependent fibrinogen bridges form between adjacent platelets, thereby promoting platelet aggregation. ADP induces the exposure of fibrinogen receptor sites on the platelet membrane. It should be noted that normal plasma fibrinogen levels support platelet aggregation. In the

absence of plasma fibrinogen, platelet fibrinogen stored in the α granules is released, promoting platelet aggregation. Platelets deficient in GP IIb/IIIa (Glanzmann's thrombasthenia) do not aggregate in response to platelet-aggregating agents.¹⁹ Platelets will not aggregate in the absence of membrane glycoproteins, fibrinogen, or divalent calcium.

The platelet membrane also includes receptors for substances such as ADP, thrombin, epinephrine, collagen, thromboxane A₂ (TXA₂), and serotonin, which play a role in platelet aggregation.¹⁵ ADP and TXA₂ are potent platelet aggregators when bound to their specific platelet membrane receptors. These two platelet-derived aggregators are released during platelet activation in response to thrombin and amplify intracellular events, leading to platelet secretion and platelet aggregation. The secretion of such substances by activated platelets causes the activation of additional platelets, thus augmenting the primary hemostatic response.

In vitro platelet aggregation can be stimulated by a variety of agents (agonists), which are listed in Box 25-3. In vitro aggregation can be visualized as a two-phase process that may be reversible or irreversible, depending on the strength of the stimulus. In the first phase, aggregation is initiated by an agonist, causing the release of small amounts of ADP from the dense granules. This initial aggregation wave is referred to as *primary* or *reversible aggregation*.¹⁹ At this point in the aggregation process, small aggregates are formed with low ambient concentrations of ADP. ADP at this concentration is only a weak platelet agonist, and therefore these aggregates may dissociate into individual platelets if the process does not proceed normally. Primary aggregation involves contraction of the circumferential microtubules and the reorganization/centralization of platelet organelles.

The second phase, or *secondary wave*, of aggregation is dependent on the activation stimulus being strong enough to evoke the secretion of platelet granule contents, particularly nonmetabolic ADP from the dense granules, as a consequence of a stronger, more complete contraction of the circumferential microtubules. The beginning of secondary aggregation actually defines the activation event of *secretion*. Ultrastructural analysis shows the internal reorganization of organelles is more severe, and degranulation is evident by the lack of density of the granules with transmission electron microscopy (TEM) (Fig. 25-9). Biochemical studies have confirmed the

BOX 25-3 Common In Vitro Platelet Aggregators

- Adenosine diphosphate* (low, optimal, and high concentrations)
- Collagen*
- Epinephrine*
- Thrombin*
- Ristocetin*
- Serotonin
- Arachidonic acid
- Immune products
- Snake venoms

*Most commonly used.

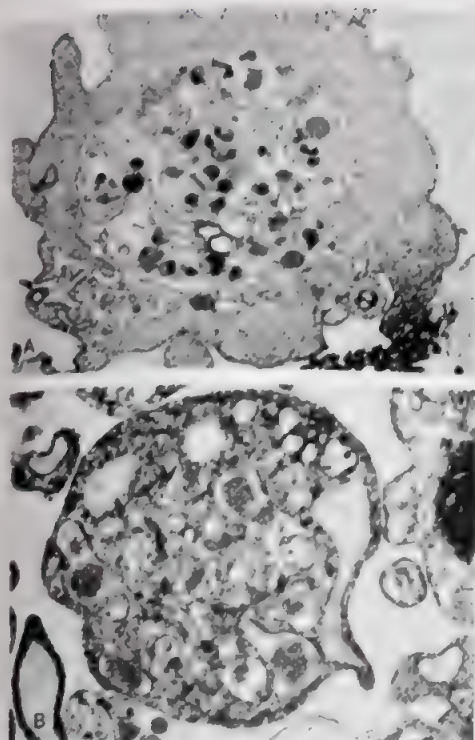


FIGURE 25-9 TEM of an activated and a degranulated platelet. **A.** Early aggregation of activated platelet (the primary wave of aggregation, a reversible process). **B.** Degranulated platelet (the secondary wave of aggregation, an irreversible process). (From Barnhart, MI. Platelet responses in health and disease. *Mol Cell Biochem.* 1978;22:117, with permission.)

release of substances such as ADP, serotonin, and epinephrine. Secondary aggregation is often considered to be irreversible.

Various laboratory methods are employed to evaluate platelet aggregation. One method of aggregometry, optical, utilizes a spectrophotometer in which platelet-rich plasma and exogenous aggregating agents are added in a cuvette. As platelets aggregate, decreasing optical density results in increased light transmittance. The change in density (percent transmittance) is recorded, creating typical aggregation "patterns" for each aggregating agent. The pattern produced is analyzed for reaction time, shape change, primary aggregation, release reaction, and secondary aggregation. Another method, lumiaggregometry, employs both optical density and fluorescence in measuring platelet aggregation and simultaneous release of ATP; firefly luciferin-luciferase reacts with the ATP producing characteristic wave patterns as the release reaction occurs. Figure 25-10 depicts a typical biphasic response of in vitro platelet aggregation to ADP, as recorded by an aggregometer. It should be noted that aggregation is an energy-dependent process that requires and exhausts the ATP resource of the platelet (see Chapter 33 for discussion of the principles, instrument methods, and interpretation of platelet aggregation).

A third method, whole blood aggregometry, measures the ability of the platelets to respond to agonists while in whole blood suspension using the electrical impedance principle. Small parallel electrodes are inserted into a cuvette containing citrated whole blood in a 1:1 saline dilution. The electrodes

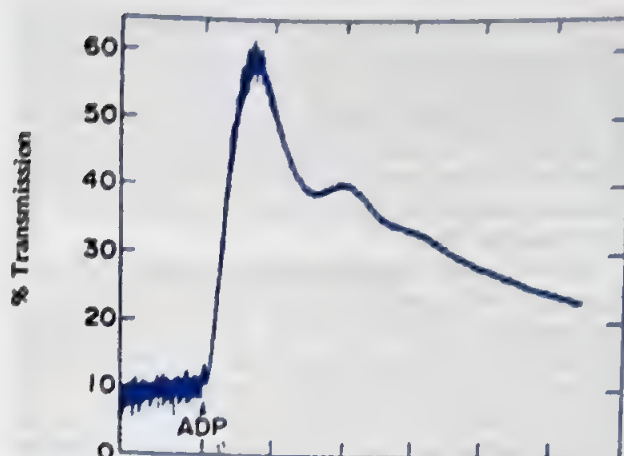


FIGURE 25-10 A typical biphasic response of in vitro platelet aggregation to ADP, as recorded via an aggregometer.

produce a small electric current running through the whole blood suspension. As platelet aggregation occurs, the platelets collect on the electrodes, reducing or impeding the magnitude of the current. The change is directly proportional to the degree of platelet aggregation. The whole blood aggregation pattern resembles a platelet-rich plasma (PRP) aggregation tracing but is measured in ohms. However, you rarely see secondary aggregation demonstrated, and the peak amplitude is much lower than in a PRP tracing. One distinct advantage of whole blood aggregation is that it virtually eliminates the need for PRP preparation, reducing the blood volume required for testing, and the platelet count does not have to be standardized. The operator pipettes an aliquot of properly mixed whole blood into the cuvette, and adds an equal volume of physiological saline prewarmed to 37°C and a stir bar. After placing the cuvette into the reaction well, the electrodes are placed into the mixture and the aggregating agent is added. The aggregation pattern is then measured.¹⁷ Recent studies have also shown that whole blood aggregation may be more sensitive, may reflect the true nature of platelet aggregation in vivo than PRP methods, and may be more sensitive to aspirin effects.^{21,22}

Secretion (the Release Reaction)

Platelet secretion (release) and secondary aggregation are intimately related and occur almost simultaneously; therefore, the discussion of the two topics is difficult to separate. Recall that the beginning of secondary aggregation actually defines the activation event of secretion. For secondary aggregation to occur, there must be secretion, particularly ADP, from the dense granules. A sufficiently strong stimulus is necessary for the secretion, or release reaction, to occur. The release reaction from dense granules involves the secretion of ADP, serotonin, and calcium. During the various stages of aggregation, platelet-secreted proteins are released from cellular organelles, thus serving as markers of platelet activation. ADP is responsible for both primary and secondary aggregation (depending on the amount secreted) and serves to amplify the process. Amplification of the initial aggregation of platelets (a reversible phenomenon) results in secondary aggregation and the

recruitment of many other platelets into a large platelet mass. The transformation of irreversibly aggregated platelets into a mass of degenerative platelet material without membranes is termed *viscous metamorphosis* (Fig. 25-11). The biochemical contents of the platelet lysosomes are also released during secretion. It is thought that these enzymes function in viscous metamorphosis and later dissolution of the platelet plug at the site of vascular injury.

Specific platelet-secreted proteins are released from α and dense granules as well as from platelet lysosomes following appropriate stimulation. Four platelet-specific proteins secreted from the α granule have been well characterized to date and are currently used as "markers of platelet activation." These include beta-thromboglobulin (β -TG), platelet factor 4 (PF4), thrombospondin, and PDGF, which confirm the degranulation of platelet α granules.¹⁶ Because β -TG, PF4, thrombospondin, and PDGF are proteins virtually absent from normal plasma and found in small concentrations within platelet α granules, these proteins specifically mark platelet activation.¹ A number of clinical conditions such as arteriosclerosis, cerebrovascular disease, cardiopulmonary bypass, shock, venous thrombosis, and DIC are associated with increased plasma levels of these markers, thus signifying platelet activation.

Stabilization of the Hemostatic Plug

The last stage involved in arresting bleeding after vessel damage is the formation of a stable platelet plug. This stabilization is achieved through the activation of the coagulation cascade and formation and deposition of **fibrin** (the end product of coagulation) on the platelet aggregates. Exposure of collagen within the subendothelium of the damaged vessel (via the intrinsic pathway) and the release of tissue factor (via the extrinsic pathway) directly initiate fibrin formation. Thus, fibrin interweaves through and over the initial platelet plug, compressing it into place at the site of the vessel injury. What originally starts as a small gelatinous mass gradually increases in size. In pathological conditions, thrombus formation may often occlude the lumen of a vessel, producing ischemia to the affected organ or tissue.¹¹ During thrombus formation, thrombin, plasminogen, tissue plasminogen activator (t-PA), and antiplasmin are all incorporated into the

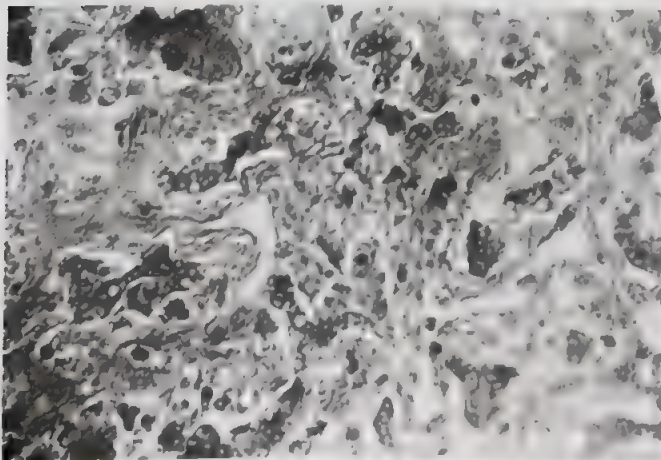


FIGURE 25-11 TEM of viscous metamorphosis.

clot.²³ As thrombin is incorporated into the clot, it is protected from degradation by its inhibitors: antithrombin (AT) and heparin cofactor II. Thrombin, now trapped within the meshwork of the clot, activates factor XIII (fibrin-stabilizing factor), resulting in the cross-linking of fibrin strands and thus, clot stabilization.

Fibrinolysis, the complex series of enzymatic reactions that promote clot dissolution, is simultaneously activated during the process of clot formation. As previously mentioned, plasminogen, the precursor of the proteolytic enzyme plasmin, is incorporated into the fibrin clot. Endothelial cells of the damaged vessel wall secrete t-PA, which catalyzes the conversion of plasminogen to plasmin.²⁴ Release of both endothelial cell t-PA and its inhibitor, plasminogen activator inhibitor (PAI), is mediated by thrombin. Any excess plasmin is controlled by its inhibitor, antiplasmin. Thus, fibrinolysis is initiated in the final phase of thrombus formation. It is important to mention that thrombin also stimulates the secretion of endothelial cell substances as well as the secretion of platelet proteins that promote tissue repair and play a major role in wound healing. Clot dissolution, therefore, provides a critical means by which formed thrombi are removed from the vascular system, blood vessels are repaired, and blood flow returns to normal. Protective mechanisms prevent thrombus formation in healthy intact blood vessels.

A review of the sequence of events involved in platelet plug formation and the approximate time involved in each stage are provided in Figure 25-12.

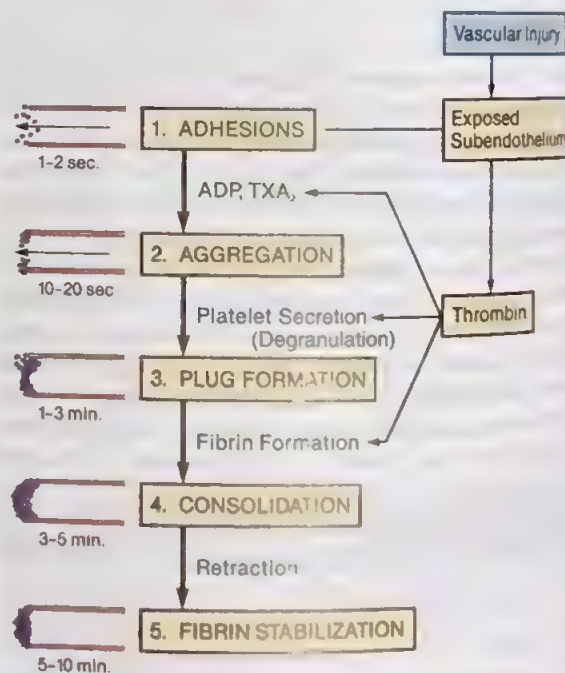


FIGURE 25-12 Sequence of events in hemostatic plug formation. (1) Platelet adhesion to exposed subendothelial connective tissue structures. (2) Platelet aggregation by ADP, thromboxane A_2 , and thrombin recruitment through transformation of discoid platelets into reactive spiny spheres that interact with one another through calcium-dependent fibrinogen bridges. (3) Contribution of platelet coagulant activity to the coagulation process, which stabilizes the plug with a fibrin mesh. (4) Consolidation of the platelet mass to provide a dense thrombus. (5) Fibrin polymerization and fibrin stabilization by factor XIII.

ADVANCED CONTENT

Binding of ADP to the platelet membrane activates phospholipase, an enzyme that cleaves the phospholipids present in the platelet membrane, freeing fatty acids such as arachidonic acid. Arachidonic acid is converted to prostaglandin endoperoxides in the cytoplasm by prostaglandin synthetase, commonly known as cyclo-oxygenase. These endoperoxides are converted to thromboxane A₂ (TXA₂), a potent platelet aggregator, mediator of platelet secretion, and a promoter of vasoconstriction. With its *in vivo* half-life of 3 seconds, TXA₂ activity is limited in time because it hydrolyzes spontaneously within the platelet to an inactive form, 11-dehydrothromboxane B₂ (11-DHTB₂).²⁴ As TXA₂ is generated with subsequent aggregating effects on platelets, calcium, sequestered in the dense tubular system of the platelet, is extruded in the sol-gel zone. Thrombin, also a potent platelet aggregator, can induce secretion of all platelet granules. Serotonin, secreted by the dense bodies of the platelet, is a weak aggregating agent as the sole stimulus but amplifies the aggregating effect of other agonists such as ADP. Serotonin serves as an important vasoconstrictor and a potent stimulator of smooth muscle prostacyclin (PGI₂) production. PGI₂ is synthesized by vascular endothelial and

smooth muscle cells from arachidonic acid. PGI₂ is a potent vasodilator and endogenous inhibitor of platelet aggregation. TXA₂ and PGI₂ are products of arachidonic acid which play a role in the regulation of the hemostatic plug and thrombus formation. (Fig. 25-13).

Effect of Aspirin on Platelet Activation

Aspirin is a widely used antithrombotic agent for the clinical treatment of arterial thrombi. Aspirin exerts a permanent yet limited effect on platelet aggregation by inhibiting the action of the cyclo-oxygenase enzyme and production of TXA₂.²⁵ Platelets are anucleated cells; thus, they lack the ability to synthesize new mRNA or protein. Exposure of platelets in the peripheral circulation to a relatively small dose of aspirin (60 to 325 mg) results in the irreversible inactivation (acetylation) of the platelet enzyme cyclo-oxygenase and inhibition of TXA₂ synthesis for the life span of the circulating platelet.²⁵ It is this quality that gives aspirin its tremendous therapeutic effect on platelet activation. Megakaryocytes, however, are capable of synthesizing cyclo-oxygenase, and newly formed platelets show normal enzyme activity. Aspirin also inhibits endothelial cell cyclo-oxygenase consistent with the fact

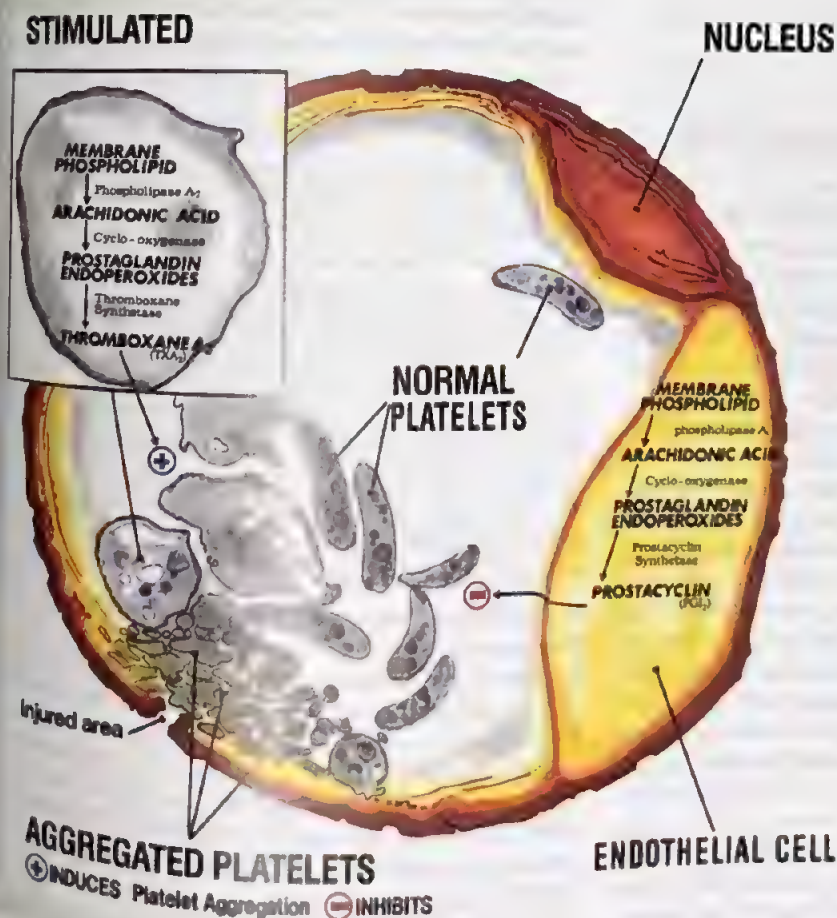


FIGURE 25-13 Synthesis of prostaglandins in platelets and endothelial cells during platelet plug formation in an injured area of a vein.

that they are genetically identical cellular products. However, endothelial cells possess the organelles necessary to synthesize cyclo-oxygenase, thereby regenerating enzyme activity as the level of circulating aspirin decreases.

The effect of aspirin on platelet cyclo-oxygenase is highly sensitive yet quite limited. Platelets activate and aggregate in response to stimuli such as thrombin and collagen. There are several other intracellular signaling pathways that mediate platelet activation and aggregation, but only one, TXA_2 synthetase, is inhibited by aspirin.²⁵ Thus, aspirin has a modest effect on platelet function in vivo, causing moderate prolongation of the bleeding time, moderate inhibition of platelet aggregation, increase in the PFA-100® closure time (epinephrine/collagen cartridge [Dade-Behring])²⁶ and reduction of the aspirin-resistance units using the arachidonic-acid cartridge with the VerifyNow™ (Accumetrics) analyzer.²⁷ Despite its limited effect on platelet function, aspirin provides a clinically significant antithrombotic effect.

CRITICAL THINKING QUESTION

25-1 von Willebrand's disease (the deficiency of von Willebrand factor) and Glanzmann's thrombasthenia (the deficiency of GP IIb/IIIa) are known bleeding disorders. Why do these two conditions cause bleeding issues?

See answers to all Critical Thinking Questions at the back of this book.

Secondary Hemostasis: Fibrin-Forming (Coagulation) System

The **fibrin-forming (coagulation) system** is the system through which coagulation factors interact to eventually form a fibrin clot. The purpose of fibrin clot formation (secondary hemostasis) is to reinforce the platelet plug (primary hemostasis). Secondary hemostasis may be started by the release of tissue factor from epithelial or endothelial cells that are exposed to the circulatory system at the site of a vascular injury. Defects in secondary hemostasis decrease fibrin production and reduce the stability of the formed clot.

This system is mediated by many **coagulation proteins (coagulation factors)** normally present in the blood in an inactive state. The coagulation factors and their most commonly used designations are listed in Box 25-4. Low levels of the coagulation factors in secondary hemostasis may be associated with bleeding that is generally delayed compared with that observed in defects in the primary hemostatic mechanism. Symptoms of defective secondary hemostasis may include soft tissue bleeding, hematomas, retroperitoneal bleeding or hemarthrosis. Good examples of these problems are the hemophilias that are associated with factor VIII and IX deficiencies (see Chapter 27). *Secondary hemostasis* is the phrase used to encompass the coagulation factors' role in the hemostatic mechanism.

Most of the coagulation factors are designated by Roman numerals. The numerical system adopted assigns the number to the factors according to the sequence of discovery and not

BOX 25-4 Nomenclature of Coagulation Factors

- Factor I - Fibrinogen
- Factor II - Prothrombin
- Factor III - Tissue thromboplastin (tissue factor)
- Factor IV - Ionized calcium (Ca^{2+})
- Factor V - Labile factor (proaccelerin)
- Factor VI - Not assigned*
- Factor VII - Stable factor (serum prothrombin conversion accelerator [SPCA] proconvertin)
- Factor VIII - Antihemophilic factor A(AHF), factor VIII:C (coagulant portion)
- Factor IX - Christmas factor (plasma thromboplastin component [PTC], Antihemophilic factor B)
- Factor X - Stuart-Prower factor
- Factor XI - Plasma thromboplastin antecedent (PTA)
- Factor XII - Hageman factor (contact factor)
- Factor XIII - Fibrin-stabilizing factor (FSF), plasma transglutaminase
- HMWK - High molecular weight kininogen (Fitzgerald factor)
- PK - Prekallikrein (Fletcher factor)

*The factor VI designation was dropped because a substance originally thought to be factor VI was found to be a precursor to factor V, and to avoid confusion, factor VI has not been reassigned.

to the point of interaction in the cascade. Some factors are routinely referred to by their common names, such as fibrinogen and prothrombin, whereas others are more commonly referred to by Roman numeral (such as factor XI, plasma thromboplastin antecedent factor).

Activation of a factor is designated by the addition of a small "a" next to the Roman numeral in the coagulation cascade (e.g., XIIa, XIa) unless convention dictates otherwise. For example, most references incorporate "thrombin" into the coagulation cascade rather than its alternate designation, factor IIa. Some of the common names are derived from the original patients who exhibited symptoms leading to elucidation of that factor deficiency and an understanding of the role of that factor in the cascade (e.g., Christmas factor, Hageman factor). Other common names describe the action of the factor in the coagulation system (e.g., fibrin-stabilizing factor).

All the coagulation proteins are produced in the liver. The von Willebrand factor portion of the factor VIII complex is produced in other body sites as well, namely, in endothelial cells and megakaryocytes.

Classification of Coagulation Factors by Hemostatic Function

In terms of general hemostatic function, the coagulation factors can be classified by hemostatic function into substrate, cofactors, and enzymes (Box 25-5)

Factor I, fibrinogen, is regarded as the main **substrate** of the blood coagulation system because the formation of a fibrin clot from fibrinogen is the final product. **Cofactors** are proteins that accelerate the enzymatic reactions involved in the coagulation process. Some examples of blood coagulation cofactors are V (labile factor) and VIII:C (antihemophilic factor [AHF]).

BOX 25-5 Classifications of Coagulation Factors by Hemostatic Function

Substrate

- Fibrinogen (factor I)

Cofactors

- Labile factor (factor V)
- Factor VIII:C (antihemophilic factor, coagulant portion)

Enzymes

- Serine proteases
 - IIa, VIIa, IXa, Xa, XIa, XIIa, prekallikrein
- Transaminase
 - Factor XIIIa

The last general category of blood coagulation factors is the enzyme category. **Enzymes** involved in coagulation can be subdivided into two groups: serine proteases and transaminases. Except for factor XIII (fibrin-stabilizing factor), all the enzymes are serine proteases when they are in their activated form. These proteases have serine as a portion of their active enzymatic site and function to cleave peptide bonds.

Factor XIII (fibrin-stabilizing factor) is the only member of the transaminase subgroup. It functions to create cross-linkages between the fibrin monomers formed during the coagulation process to produce a stable fibrin clot.

Classification of Coagulation Factors by Physical Properties

On the basis of their physical properties, the coagulation proteins can be divided into three groups: the contact proteins, the prothrombin proteins, and the fibrinogen or thrombin-sensitive proteins (Table 25-7).

The **contact protein group** includes factor XII (Hageman factor), FXI, prekallikrein (PK; Fletcher factor), and high molecular weight kininogen (HMWK; Fitzgerald factor). These proteins are involved in the initial phase of intrinsic system activation. Although deficiencies of these coagulation proteins are associated with markedly abnormal laboratory tests, a bleeding tendency is not an issue. Interestingly, problems with thrombosis have been reported in patients with factor XII (Hageman factor) and prekallikrein (Fletcher factor) deficiencies. The contact group works to activate factor XI.

The **prothrombin group** (see Table 25-7) generally consists of low molecular weight proteins that include factors II (prothrombin), VII (stable factor), IX (Christmas factor), and X (Stuart-Prower factor). Each member of this group contains a unique amino acid: gamma carboxyglutamic acid, which is necessary for both calcium binding and attraction of these coagulation factors to the surface of activated platelets, where the formation of a fibrin clot occurs. Because vitamin K is required for the carboxylation of the glutamic acid residues, this group is also known as the vitamin K-dependent coagulation proteins. These factors may also be selectively removed from plasma by adsorption on barium sulfate (BaSO_4).

TABLE 25-7 Classification of Coagulation Proteins by Physical Properties

Physical Properties	Coagulation Proteins		
	Contact Group	Prothrombin Group	Thrombin-Sensitive Group
Factors	XI, XII, PK, HMWK	II, VII, IX, X	I, V, VIII, XIII
Consumed during coagulation	No	No (except II)	Yes
Present in serum	Yes	Yes (except II)	No
Present in stored plasma	Yes	Yes	No*
Adsorbed by BaSO_4	No	Yes	No
Vitamin K-dependent	No	Yes	No

*Factors V and VIII are not present in stored plasma because of their labile nature, but factors I and XIII are present. PK = prekallikrein; HMWK = high molecular weight kininogen; BaSO_4 = barium sulfate

Drugs that act as antagonists to vitamin K (such as warfarin [Coumadin], commonly used for oral anticoagulant therapy) inhibit this vitamin K-dependent reaction, which is required for functionally active coagulation factors of the prothrombin group.¹⁰ Factors II, VII, IX, and X; proteins C and S; and protein Z are still synthesized by the liver but are nonfunctional because they lack the specific receptors for calcium and cannot enter into the formation of an enzyme-substrate complex.²¹ Therefore, patients who are nutritionally vitamin K-deficient also exhibit decreased production of functional prothrombin proteins. These dysfunctional factors are known as "Proteins Induced by Vitamin K Absence or Antagonists."

Acquired deficiencies of the vitamin K-dependent coagulation factors are relatively common because the body does not contain appreciable stores of vitamin K. Clinical situations in which a vitamin K deficiency can develop include patients who have just had surgery and are receiving parenteral feeding, patients who are receiving high doses of intravenous antibiotics, and patients suffering from liver disease.

The **fibrinogen group** (see Table 25-7) consists generally of high molecular weight proteins that include factors I (fibrinogen), V (labile factor), VIII (antihemophilic factor), and XIII (fibrin-stabilizing factor). During coagulation, generated thrombin acts on all the factors in the fibrinogen group. Thrombin enhances the activity of factors V and VIII by converting these proteins into active cofactors that are involved in the assembly of macromolecular complexes on the surface of activated platelets. Thrombin converts fibrinogen to fibrin and activates factor XIII.

Factors V and VIII:C are the least stable factors because their activity is relatively labile to degradation and

denaturation. Therefore, testing for factor V and VIII:C should be rapid, unless appropriate storage measures are taken. In addition to their presence in plasma, these factors are also found within platelets. Some factors within the fibrinogen group have been reported to increase with inflammation, pregnancy, and with the use of oral contraceptives. Other physical properties of the coagulation factors are summarized in Table 25-8.

Blood Coagulation: The "Cascade" Theory

The process of blood coagulation involves a series of biochemical reactions that transforms circulating substances into an insoluble gel through conversion of soluble fibrinogen to fibrin. This process requires plasma proteins (coagulation factors) as well as phospholipids and calcium.

Blood coagulation leading to fibrin formation can be separated into two pathways, extrinsic (Fig. 25-14) and intrinsic (Fig. 25-15), both of which share specific coagulation factors with the common pathway (Fig. 25-16).²⁹ Both extrinsic and intrinsic pathways require initiation, which leads to subsequent activation of various coagulation factors in a cascading, waterfall, or domino effect. Useful demonstrations can be derived from the waterfall or domino concept. According to the cascade theory, each coagulation factor is converted to its active form by the preceding factor in a series of biochemical chain reactions. Calcium (Ca^{2+}) participates in some of the reactions as a cofactor. Each reaction is promoted by the preceding reaction.

Eventually, both the extrinsic and intrinsic systems enter the common pathway with generation of the enzyme thrombin, which converts fibrinogen to fibrin (Figs. 25-17 and 25-18). The term *extrinsic* is used because this pathway is initiated when tissue factor, a substance not found in blood, enters the vascular system (see Fig. 25-14). The tissue factor includes a phospholipid component that is the source of the required phospholipid in the extrinsic system (Fig. 25-19). Phospholipid provides a surface for interaction of various factors. The phospholipids required in the intrinsic pathway are provided by the platelet membrane. In the intrinsic pathway, all the factors necessary for clot formation are intrinsic to the vascular compartment because they are all found within the circulating blood (see Fig. 25-15).

Extrinsic Pathway (Factor VII)

In the extrinsic pathway, factor VII is activated to factor VIIa in the presence of Ca^{2+} (factor IV) and the tissue factor (factor III), which is released from the injured vessel wall (see Fig. 25-14). Only factor VIIa, Ca^{2+} , and factor III (tissue factor) are needed to activate factor X to Xa.

The extrinsic pathway provides a means for very quickly producing small amounts of thrombin, leading to fibrin formation (see Figs. 25-17 and 25-18). In addition, the VIIa-tissue factor complex can activate factor IX to IXa in the intrinsic pathway. In the laboratory, the prothrombin time (PT) test is used to monitor the extrinsic pathway (for a review of the procedure, see Chapter 33).

TABLE 25-8 Physical Properties of the Coagulation Factors

Factor	Clotting Pathway	Molecular Weight (kD)	Half-Life in Vivo (h)	Plasma Concentration (mg/dL)	Storage Stability
I	Intrinsic, extrinsic, common pathway	340	90-150	200-400	Stable
II	Intrinsic, extrinsic, common pathway	70	50-100	10-15	Stable
III	Extrinsic system only	45	—	0	Stable
V	Intrinsic, extrinsic, common pathway	330	12-36	0.5-1.2	Labile
VII	Extrinsic system only	48	4-6	0.05-2.0	Stable
VIII/vWF	Intrinsic system only	350	8-12	0.01-0.1	Labile
IX	Intrinsic only	60	20-24	0.3-0.4	Stable
X	Intrinsic, extrinsic, common pathway	59	24-65	0.5-1.0	Stable
XI	Intrinsic only	160	40-80	0.5-1.2	Stable
XII	Intrinsic only	80	50-70	3.0-4.0	Stable
XIII	Intrinsic, extrinsic, common pathway	320	72-150	1.0-2.5	Stable
PK	Intrinsic only	85	35	3.0-5.0	Stable
HMWK	Intrinsic only	150	150-160	0.01-0.1	Stable

Note: all factors are present in normal fresh plasma.
PK = prekallikrein, HMWK = high molecular weight kininogen.

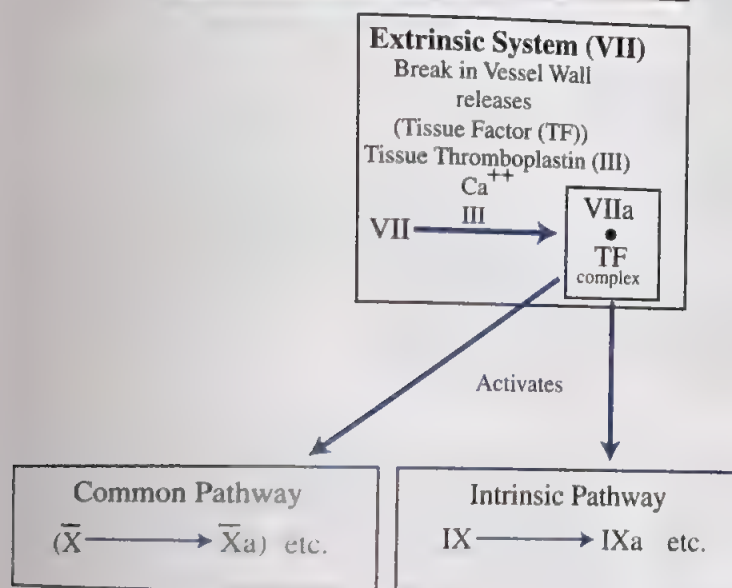
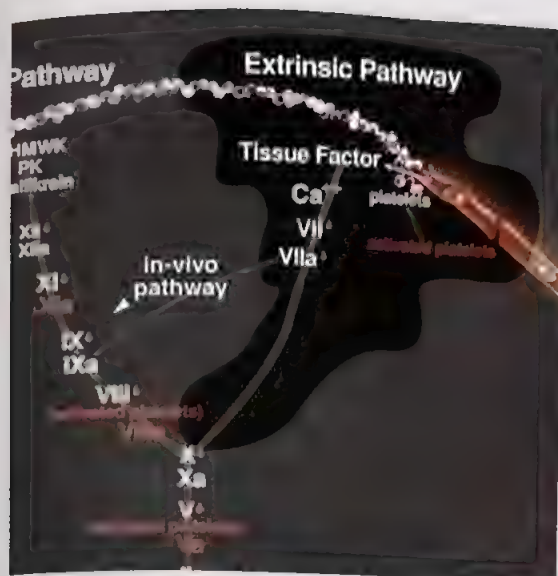


FIGURE 25-14 The extrinsic pathway and the role of factor VIIa in activation of factors X and IX (From American Bioproducts Company. Modified with permission.)

Intrinsic Pathway (Factors XII, XI, IX, and VIII)

Subsequent exposure to negatively charged foreign substances such as collagen, subendothelium, or phospholipids, activation of factor XII involving "contact factors" and factor XI initiate clotting through the intrinsic pathway (see Fig. 25-15).

Once generated, factor XIIa, in the presence of Fitzgerald factor (HMWK) and Fletcher factor (prekallikrein), converts factor XI to XIa. Factor XIIa is capable of activating factor XI without HMWK, but this activation takes place much more slowly.³⁰

Deficiencies in these proteins (prekallikrein, HMWK, and factor XII) are not associated with bleeding tendencies. Factor XIIa deficiency is not known to cause bleeding complications; rather, it is associated with paradoxical fatal thromboembolic complications.³¹

The next reaction in the intrinsic pathway is the activation of factor IX to factor IXa by factor XIa, in the presence of Ca^{2+} . Activated factor IX (IXa) participates, along with the essential cofactor VIII:C, in the presence of ionized calcium and PF3, a source of phospholipids, to activate factor X, which leads to the generation of thrombin and formation of fibrin. The complex consisting of factor IXa-factor VIIIa-phospholipid- Ca^{2+} has been called the *tenase complex* because it activates factor X (Fig. 25-20).³²

The macromolecular complex of factors IXa, VIIIa, X, PF3, and Ca^{2+} assembles on the surface of the activated platelet (providing the phospholipid) during the intrinsic pathway of blood coagulation.²⁶ This surface provides a protective environment that facilitates the enzymatic reactions of the coagulation cascade without interference from the physiological anticoagulants normally present in plasma.

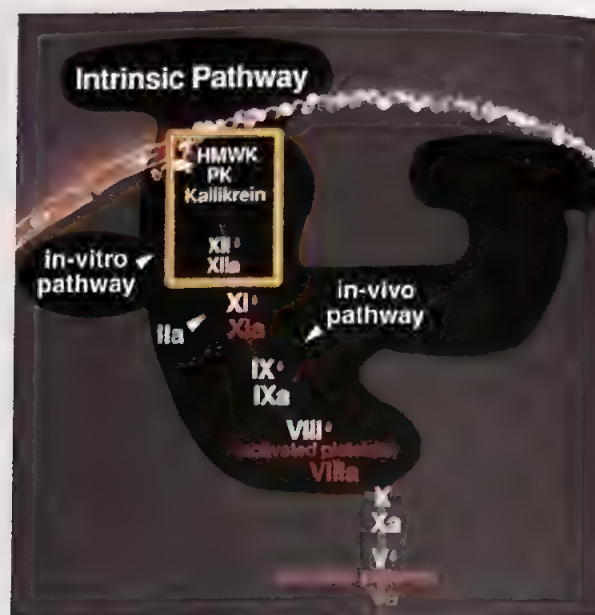
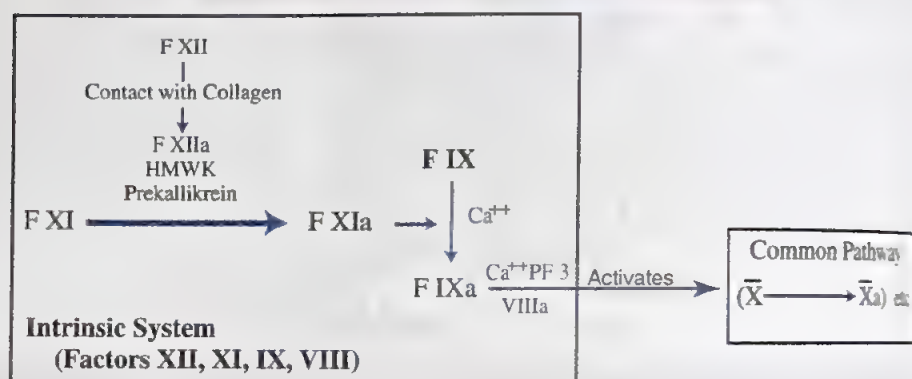


FIGURE 25-15 The intrinsic pathway and its role in activation of factor X. HMWK = high molecular weight kininogen; PF3 = platelet factor 3. (From American Bioproducts Company. Modified with permission.)



In regard to the intrinsic pathway, it is also important to be familiar with the properties of the factor VIII complex (Box 25-6). Factor VIII complex consists of two main portions, factor VIII:C (the procoagulant protein) and vWF (the carrier protein).

It should be noted that factor VIII requires enhancement by the generated enzyme thrombin to amplify its activity.³⁰ In the laboratory, the activated partial thromboplastin time (aPTT) test is used to evaluate the intrinsic pathway. During laboratory testing, the intrinsic pathway is initiated in vitro by activation on negatively charged surfaces, such as glass or kaolin, or chemically by ellagic acid (for a review of this procedure, see Chapter 33).

The factor VIII complex, which consists of several components, comprises the largest protein involved in the coagulation cascade. The major portion of this protein complex is considered to be the carrier protein called *von Willebrand factor (vWF)*, although this is not the portion active in the coagulation cascade. A smaller subunit or protein that is associated with factor VIII is responsible for the clotting or procoagulant activity of factor VIII (VIII:C). The C in the expression VIII:C stands for "coagulant." It is factor VIII:C that is functionally active in the coagulation cascade.

The vWF portion of the complex carries the VIII:C procoagulant portion. The vWF portion may exhibit a stabilizing effect over factor VIII:C by protecting it from proteolytic activity.¹² Because of vWF's extremely large size and its ability to bind to the platelet membrane Ib/IX/V and IIb/IIIa receptors, it appears to have a role in anchoring the platelet plug to the vessel breach.²³

ADVANCED CONTENT

Three physiological triggers of the intrinsic pathway have been described: collagen, polyphosphates, and neutrophil extracellular traps. Ca⁺⁺ and platelet-derived polyphosphates are able to activate F XII, triggering coagulation factors further downstream. Polyphosphate-dependent F XII activation does not appear to lead to a faster clot formation but rather to increased fibrin clot stability. This could be the reason why high levels of F XII associate with thromboses while F XII deficiency may cause the clot to be unstable and lead to embolization.³²

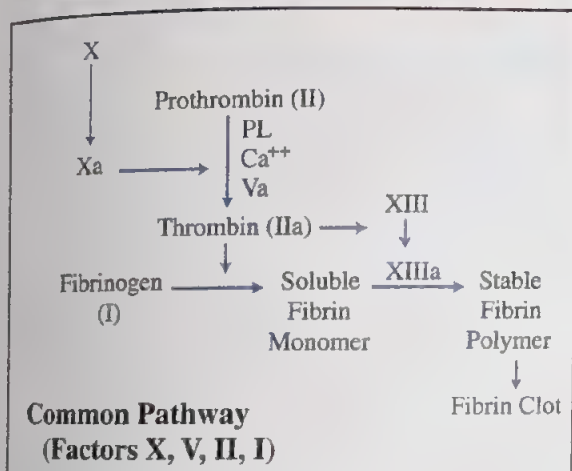


FIGURE 25-16 The common pathway and formation of fibrin clot. PL = phospholipid source. (From American Bioproducts Company. Modified with permission.)

Common Pathway (Factors X, V, II, and I)

The terms *intrinsic* and *extrinsic* pathways really do not fit with the current understanding of hemostasis; however, they are still used to identify the coagulation factors. The common pathway begins with the activation of factor X of the intrinsic system, the extrinsic system, or both (see Figures 24-25 and 25-17). Factor Xa, in the presence of factor V and a phospholipid (PF3), converts prothrombin to its active form, thrombin. Thrombin then takes the following actions: it feeds back to activate factors VIII and V, converts fibrinogen to soluble fibrin monomer, and helps to stabilize the fibrin monomer by converting factor XIII to XIIIa, which cross-links the fibrin monomers to form stable fibrin polymer. Thrombin also has other actions (see later discussion in the section Thrombin-Mediated Reactions in Hemostasis). Because the common pathway contains the factors X, V, II, and I, these factors may be monitored by both the PT and the aPTT.

From the extrinsic system, factor VIIa in the presence of tissue factor and ionized calcium converts factor X to Xa. From the intrinsic system, the tenase complex (factor IXa in the presence of factor VIII:C, phospholipid [PF3], and Ca^{2+}) converts factor X to Xa (see Figs. 25-17 and 25-18).

After the formation of factor Xa, this activated factor, along with cofactor V, in the presence of Ca^{2+} and phospholipid, converts factor II, prothrombin, to the active enzyme thrombin (IIa). The phospholipid is present to provide surfaces so that prothrombin and factor X can be bound by bridges of ionized calcium.¹⁵ The association of factor Xa, factor V, phospholipid, and Ca^{2+} is called the **prothrombinase complex** (or the prothrombin activator) because it enzymatically converts the substrate prothrombin to the enzymatically active thrombin (Fig. 25-21).²³ This additional macromolecular complex of factors Xa, Va, Ca^{2+} , phospholipid, and prothrombin also assembles on the surface of activated platelets.

Activation of thrombin is slow, but once generated, it further amplifies coagulation by converting fibrinogen to fibrin, activating factor XIII, enhancing the activity of factors V and VIII, and inducing platelet aggregation. Thrombin acts on fibrinogen to form fibrin monomers. Fibrinogen is composed of three pairs of polypeptide chains (two alpha α chains, two beta β chains, and two gamma [γ] chains). Thrombin cleaves a portion of each of the α and β polypeptides to form fibrinopeptides A and B. Most of the α and β chains are still left on the fibrinogen molecule. After this cleavage of fibrinopeptides A and B, the remainder of the fibrinogen molecule is called the **fibrin monomer** (Fig. 25-22).

By using the PT and aPTT test results in the laboratory, one can identify defects or deficiencies as occurring in the intrinsic, extrinsic, or common pathways of blood coagulation with the exception of factor XIII functional deficiency.

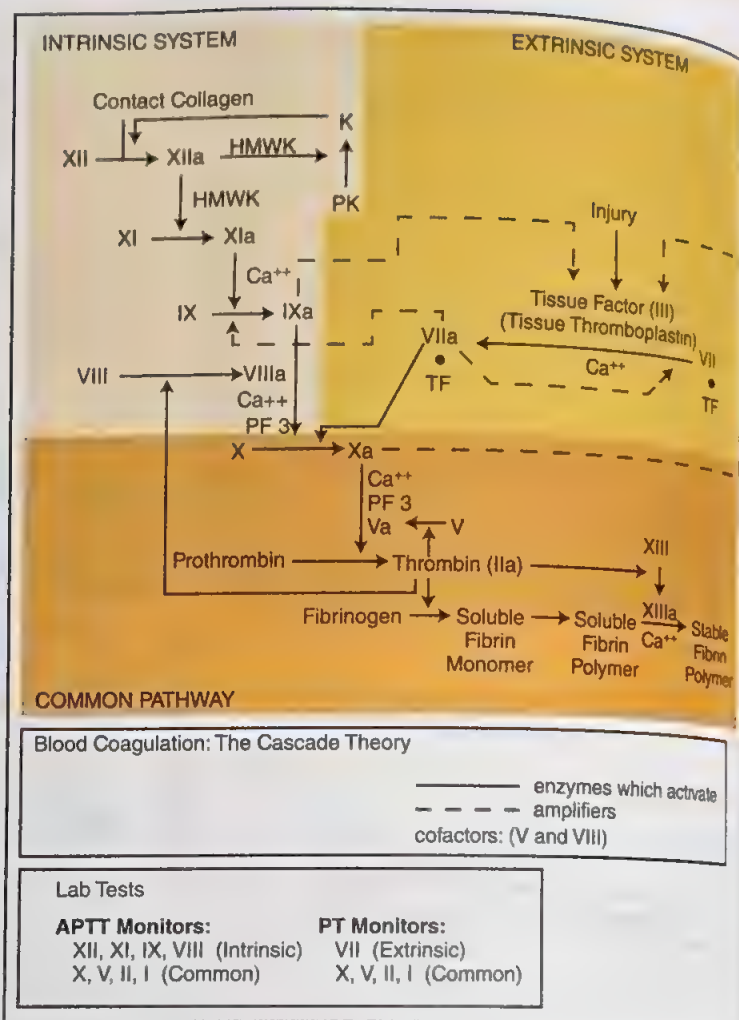
Thrombin-Mediated Reactions in Hemostasis

Each of the four major components of coagulation and the various substances produced by each component help to regulate the basal state of hemostasis by which blood remains fluid and cell surfaces remain nonthrombogenic. Vascular damage initiates physical, cellular, and molecular changes to platelets, endothelium, and circulating coagulation proteins. When the circulating factors of coagulation become activated, they factors possibly bind to these activated factors, thus coagulating the blood to halt response. Numerous pathways exist to regulate hemostasis by which the body can respond and collectively prevent the formation of a blood clot. The body can respond by activating the cellular components of hemostasis to help regulate the basal state of hemostasis. The body can respond by activating the circulating factors of hemostasis to help regulate the basal state of hemostasis. The body can respond by activating the cellular components of hemostasis to help regulate the basal state of hemostasis.

Thrombin-Mediated Platelet Aggregation

Thrombin is considered to be a potent platelet-aggregating agent. The binding of thrombin to specific platelet membrane receptors initiates cellular events leading to platelet

FIGURE 25-17 Blood coagulation: The “cascade” theory of coagulation. The extrinsic system, the intrinsic system, and the common pathway and the appropriate laboratory tests for evaluation of each. HMWK = high molecular weight kininogen; PF3 = platelet factor 3; PK = prekallikrein; PT = prothrombin time; APTT = activated partial thromboplastin time.



secretion and aggregation. Thrombin promotes secretion of serotonin, a vasoconstrictor, and TXA_2 , a platelet-aggregating agent. As a result of thrombin-induced secretion, vessels constrict, limiting blood flow, and platelets aggregate. The hemostatic plug grows in size, eventually being enmeshed within fibrin. Activated platelets provide surface phospholipids for the assembly of coagulation factors and further thrombin generation.

▶ ADVANCED CONTENT

When platelets are activated, phosphatidylserine (PS) in the inner leaflet of the platelet membrane is transported to the outer leaflet of the platelet membrane. Scott syndrome is a rare autosomal recessive inherited disease caused by *TREMI6F* (*ANO6*) mutation. The mutation results in defective translocating PS to the platelet membrane and reduced thrombin generation, and the patients often present with isolated bleeding after surgery or trauma, menorrhagia, and postpartum hemorrhage. Platelet numbers, morphology, adhesion, secretion, and aggregation appear normal.³³

Thrombin Formation: Role of Extrinsic Pathway

Thrombin's main duty is to cleave fibrinopeptides A and B from the alpha and beta chains of the fibrinogen molecule, thus triggering fibrin polymerization. Also, thrombin amplifies the coagulation mechanism by activating cofactors V, VIII, and factor XI.²³ Formation of thrombin occurs by way of the extrinsic pathway and tissue factor. Tissue factor, a membrane glycoprotein, is released from cells and tissues and binds to factor VII in circulation, thus activating factor VII to factor VIIa. The TF-VIIa complex then activates either factor IX or X. The prominent pathway is thought to be activation of factor IX to promote coagulation and fibrin formation.

Thrombin Formation: Role of Common Pathway

Factors X, II (prothrombin), I (fibrinogen), and cofactor V are critical to the formation of a thrombus in circulation. Activated factor IXa (via factor VIIa), factor X, Ca^{2+} , and thrombin-activated VIIIa assemble on membrane platelet phospholipid and catalyze the activation of factor X. Newly generated factor Xa, membrane-bound thrombin-activated factor Va, Ca^{2+} , and factor II (prothrombin) assemble on the platelet membrane, forming the prothrombinase complex.

Coagulation Cascade

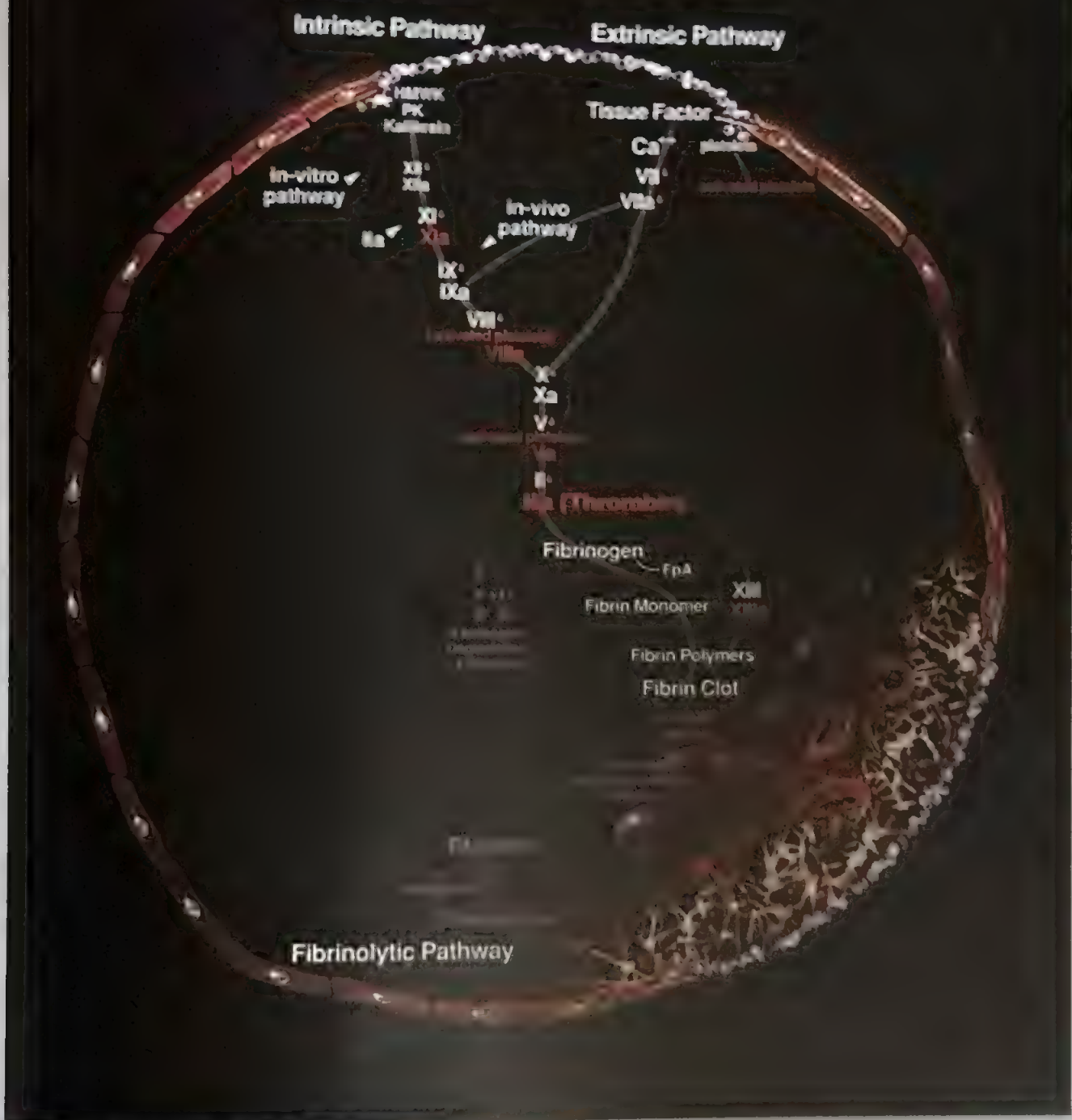


FIGURE 25-18 An overview of coagulation cascade, the intrinsic and extrinsic pathways, and the interaction between the two. The fibrinolytic pathway and its action on fibrinogen and fibrin. HMWK = high molecular weight kininogen; PK = prekallikrein; FpA = fibrinopeptide A. (From Diagnostica-Stago, Inc. with permission.)

This complex catalyzes the conversion of prothrombin to thrombin. Thus, this thrombin-modulated pathway provides a positive feedback mechanism to amplify the generation of thrombin from prothrombin at a faster rate. Increased thrombin concentrations serve to amplify the activation of cofactors V and VIII, which, in turn, leads to enhanced thrombin formation from its precursor prothrombin.

Thrombin-Mediated Anticoagulant Activity

Antithrombin (AT), formerly known as AT III, is the main physiological inhibitor of thrombin; factors Xa, IXa, XIa, and XIIa; activated protein C; and kallikrein (Fig. 25-23). In the presence of heparin, the inactivation of thrombin and factor Xa by AT is significantly increased. AT is consumed in the inhibition of thrombin.

Phospholipid, Ca^{++} -Dependent Reactions of Extrinsic and Common Pathway

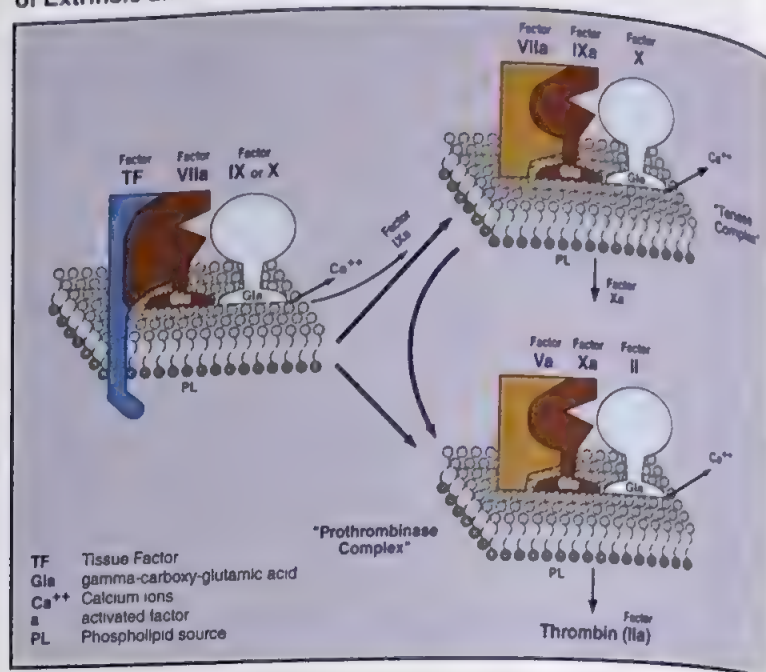


FIGURE 25-19 Platelet membrane phospholipid provides a surface for the interaction of coagulation factors and the formation of "tenase complex" and "prothrombinase complex."

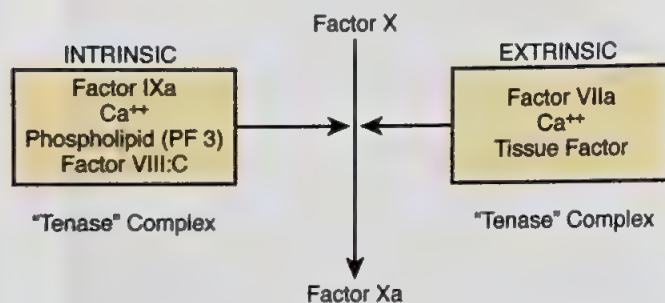


FIGURE 25-20 Activation of factor X at the beginning of the common pathway and the "tenase" complex.

ADVANCED CONTENT

Fondaparinux is a low molecular weight heparin that inhibits thrombin formation by inhibiting factor Xa. The chromogenic antifactor Xa assay is currently the gold standard for monitoring low molecular weight heparin (LMWH) and fondaparinux therapy.³⁴

The role of protein C (a vitamin K-dependent factor) as an anticoagulant is related to the presence of thrombin, thrombomodulin, and protein S.³⁵ **Protein C** is activated by thrombin and endothelial cell thrombomodulin. The formation of the thrombin-thrombomodulin complex accelerates the activation of protein C. Activated protein C exerts an anticoagulant effect by inactivating cofactors Va and VIIIa, thus slowing the rate of thrombin formation.³⁶ It is important to note that activated protein C does not inhibit the other regulatory roles of

BOX 25-6 Factor VIII Complex

Smaller Protein Subunit

- Nomenclature
 - VIII (referring to the procoagulant portion)
 - VIII:C (for coagulant)
- Components
 - VIII:C/Ag—antigenic determinant of VIII, measured by immunoassays with human antibodies to VIII
 - VIII:C—procoagulant property of normal plasma measured in the activated partial thromboplastin time (aPTT) test as coagulant activity
- Characteristics
 - Inherited as sex-linked recessive
 - Acts as a cofactor in a complex with factor IXa, Ca^{++} , and PF3 to activate factor X to Xa

Major Protein Portion

- Nomenclature
 - vWF (von Willebrand factor)
 - VIII:vWF
- Components
 - vWF:Ag—antigenic determinant on vWF that is detected by using heterologous antibodies to vWF
 - Ristocetin cofactor (VIII:Rco)—the property of normal plasma vWF that supports ristocetin-induced agglutination of washed normal platelets
- Characteristics
 - Usually inherited as autosomal dominant
 - Responsible for platelet adhesion
 - Responsible for ristocetin-induced aggregation of platelets
 - Stabilizes VIII:C when bound to vWF during circulation, and functions in protection of VIII:C from proteolytic inactivation or removal from the circulation

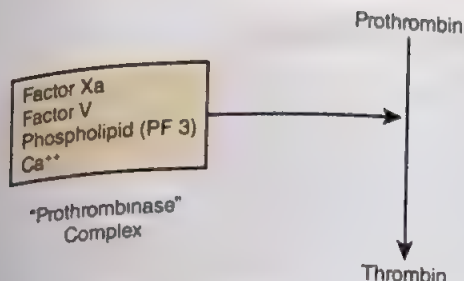


FIGURE 25-21 Conversion of prothrombin to thrombin by prothrombinase complex.

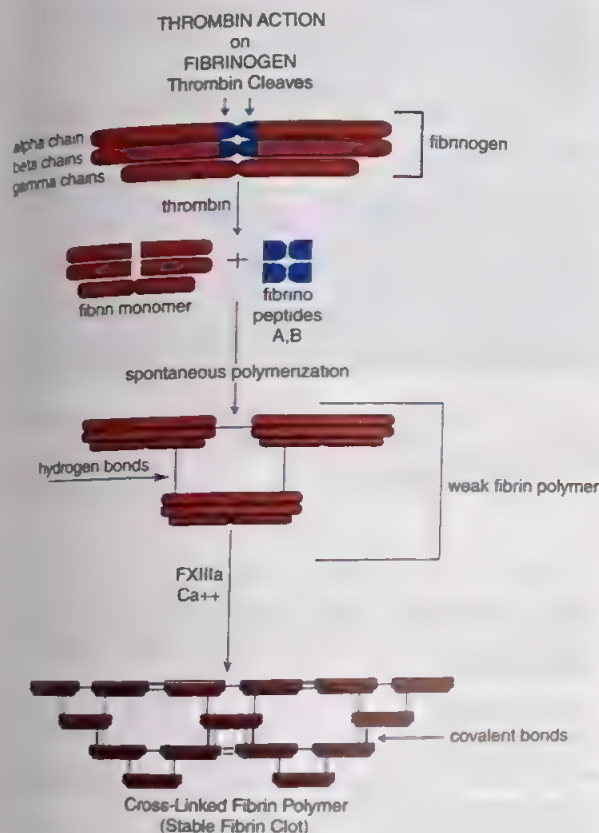


FIGURE 25-22 Thrombin activity on fibrinogen.

thrombin in hemostasis. The inhibition of cofactors Va and VIIIa by protein C is enhanced by the presence of protein S, another vitamin K-dependent factor. Activated protein C is also inhibited by other plasma proteins. The role of activated protein C is to turn off the amplification pathway of thrombin generation. It is also interesting to note that the reactions that lead to thrombin formation from its precursor prothrombin are regulated in part by thrombin-mediated mechanisms (see Chapter 29 for a detailed discussion of anticoagulant therapy).

Thrombin-Mediated Tissue Repair

Thrombin has numerous effects on the cells that play a role in tissue repair and wound healing. Shortly after thrombin generation, there is increased vascular permeability and increased

BOX 25-7 Thrombin-Mediated Reactions in Hemostasis

Procoagulant

- Induces platelet activation and aggregation
- Activates cofactor VIII to VIIIa
- Converts fibrinogen to fibrin
- Activates factor XIII to XIIIa
- Via autocatalysis converts prothrombin to thrombin

Coagulation Inhibitor

- Binds with antithrombin to inhibit serine proteases (XIIa, XIa, Xa, IXa, and kallikrein)
- Promotes endothelial cell release of t-PA
- Binds to thrombomodulin to activate protein C (inhibits Va and VIIIa)

Tissue Repair

- Induces cellular chemotaxis
- Stimulates proliferation of smooth muscle and endothelial cells

adhesion of leukocytes to endothelial cells mediated by various adhesion molecules secreted by platelets and endothelial cells. Neutrophils and monocytes undergo chemotaxis in response to thrombin. Thrombin mediates the release of a potent smooth muscle mitogen (PDGF [platelet-derived growth factor]) as well as stimulates the proliferation of fibroblasts, smooth muscle cells, and endothelial cells, thus aiding in vascular repair.

Fibrin-Lysing (Fibrinolytic) System

The fibrin-forming and fibrin-lysing systems are intimately related. Activation of coagulation also activates fibrin lysis. **Fibrinolysis**, the physiological process of removing unwanted fibrin deposits, represents a gradual progressive enzymatic cleavage of fibrin to soluble fragments. These fragments are then removed from the circulation by the fixed macrophages of the mononuclear phagocytic system. This action of the fibrinolytic system reestablishes blood flow in vessels occluded by a thrombus and facilitates the healing process following injury. For a detailed description of the fibrinolytic system refer to Chapter 28.

The kinin system is a minor system involved in the hemostasis process. This system is activated by both the coagulation and fibrinolytic systems and is important in inflammation, vascular permeability, and chemotaxis.

Prekallikrein (PK) and **high molecular weight kininogen (HK)** are additionally needed to enhance or amplify the contact factors involved in the intrinsic system (Fig. 25-24). Specifically, factor XIIa in the presence of HK converts prekallikrein to kallikrein. Kallikrein feeds back to accelerate the conversion of factor XII to XIIa, speeding up intrinsic system processes.

Antithrombin (AT) and Protein C Pathways

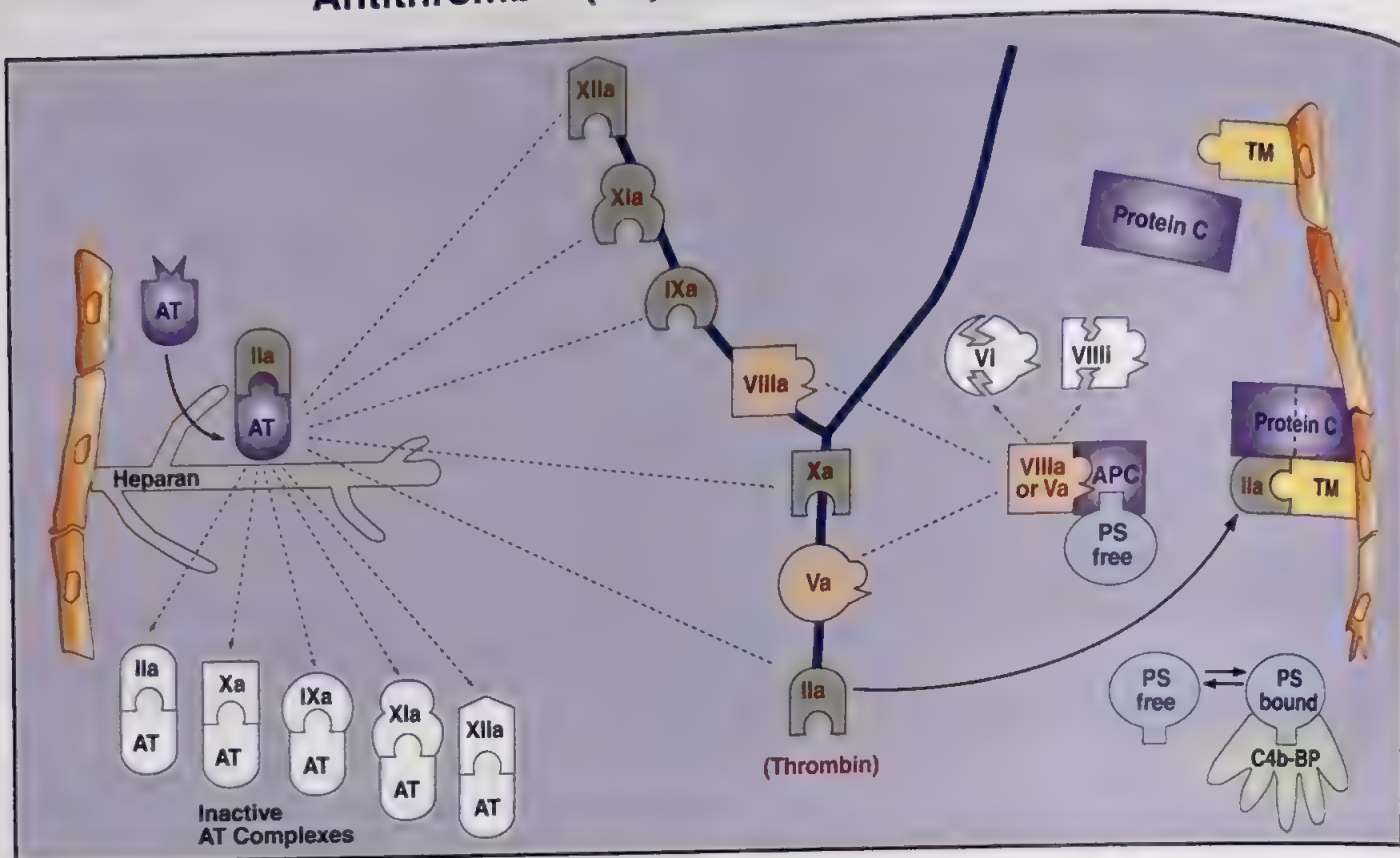


FIGURE 25-23 The inhibitor pathway of coagulation. AT = antithrombin; PC = protein C; APC = activated protein C; PS = protein S; C4b-BP = C4b-bound protein; TM = thrombomodulin.

The activation of factor XII acts as the common link between many aspects of the hemostatic mechanism, including the fibrinolytic system, the kinin system, and the complement system (see Chapters 27 and 28).

Complement System

The complement system is composed of approximately 22 serum proteins that, working together with antibodies and clotting factors, play an important role as mediators of both immune and allergic reactions. The reactions in which complement participates take place in the blood or in other body fluids. The most important biological role of complement is the production of cell membrane lysis of antibody-coated target cells. Two independent pathways of activation of the complement cascade may occur along with a common cytolytic pathway. These are designated the classic and alternate pathways of complement activation.

Both the coagulation system and the fibrinolytic system are interrelated with the complement system.³⁷ The interrelationship of the coagulation, complement, and fibrinolytic systems is discussed in Chapter 28.

Laboratory Evaluation of Hemostasis

The diagnosis of any hemostatic disorder is made by the systematic evaluation of information obtained in the history and

physical examination, along with the appropriate laboratory testing. Diagnostically, the most valuable data from a patient's history include:

- Documentation of the physical appearance, site, severity, and frequency of bleeding episodes
- A reliable patient and family history of bleeding disorders
- An accurate drug history
- Other contributing or underlying illnesses

Bleeding disorders present themselves differently, depending on the causative problem. Two general rules apply: first, patients with platelet disorders usually exhibit petechiae and mucous membrane bleeding. In general, this is because a defect of primary hemostasis is present, resulting in the formation of a defective platelet plug. Second, patients with coagulation defects may develop deep spreading hematomas and bleeding into the joints with evident hematuria. In general, this is because a defect of secondary hemostasis is present, resulting in the inadequate fibrin reinforcement of a functionally normal platelet plug.

Alteration of any aspect of the hemostatic mechanism may cause abnormal bleeding in a wide variety of familial and acquired clinical disorders. These defects may be classified into three broad categories that can be diagnostically approached by a systematic laboratory evaluation. These include vascular and platelet disorders, coagulation

The aPTT test measures factors of the intrinsic and common pathways of blood coagulation (XII, Fletcher, Fitzgerald, XI, IX, VIII, X, V, II, and I). It should be noted that factors XII, XI, IX, VIII, Fletcher, and Fitzgerald are limited to the intrinsic system. Deficiencies or inhibitors of any of these factors will result in an abnormally prolonged aPTT. Both the PT and aPTT tests will show prolonged results with an abnormality of the shared factors of the common pathway (X, V, II, and I). Typically, the aPTT will only be prolonged in fibrinogen (factor I) deficiencies if the level is less than 100 mg/dL. A factor abnormality refers to a deficiency of that factor in plasma for any one of the following reasons:

- Decreased synthesis
- Synthesis of a dysfunctional factor molecule
- Excessive destruction of factors through acquired disorders
- Inactivation of factors through circulating inhibitors

Neither the PT nor the aPTT screens for factor XIII activity. The PT and aPTT assays test for the initial conversion of fibrinogen to fibrin. Cross-linked stabilized fibrin, which develops later through mediation of factor XIIIa, does not have an effect on the PT or aPTT. Special testing to assess factor XIII activity must be done (see Chapter 33).

PT and aPTT testing has been reported in a variety of ways, such as patient seconds and control seconds. Past reporting, using ratios for PT testing and percentage, has largely been abolished. The International Normalized Ratio (INR) is the method of choice for PT reporting, because it adjusts for source-related thromboplastin sensitivity differences and testing methodology through use of a mathematical exponent, the International Sensitivity Index (ISI).³⁸ The ISI is unique to each batch of thromboplastin/instrumentation combination and is furnished by the manufacturer.

Numerous articles state the method for reporting INR values. INR standardizes PT reporting worldwide by adjusting all reported values to a World Health Organization international reference thromboplastin standard. Therefore, all PT results reported by INR methodology are theoretically comparable. Using the INR values facilitates optimal oral anticoagulant therapy in patients at risk for thrombosis, especially those on warfarin who travel extensively, and requires frequent monitoring. Thromboplastins with a low ISI (less than 1.2) correlate better to recombinant thromboplastin, which replaced

human-brain thromboplastin for standardizing INR values by the World Health Organization.³⁹

However, PT and aPTT are poor predictors of bleeding during invasive procedures. Thromboelastography (TEG) measures global hemostasis and fibrinolytic function, which includes interaction of primary and secondary hemostasis. TEG has the capability to assess platelet function, clot strength, fibrinolysis, and heparin-associated anticoagulation with a quick turnaround time. TEG is being used to predict the need for blood products and certain medications in patients who are bleeding.⁴⁰

The **thrombin time** is a measure of the ability of thrombin to convert fibrinogen to fibrin and is particularly useful in the evaluation of circulating anticoagulants (pathological inhibitors). The thrombin time is prolonged in the following conditions:

- Hypofibrinogenemia and dysfibrinogenemia
- Treatment with heparin
- Circulating FDPs
- Pathological circulating inhibitors

Additional laboratory testing is designed to narrow down the abnormality to one of these specific areas. As a result, laboratory testing can be divided into categories:

- Screening tests for vascular or platelet dysfunction (such as PFA-100, platelet aggregation using platelet-rich plasma or whole blood, and PF3 assay)
- Tests for coagulation (such as factor assays)
- Special tests (e.g., for fibrinolytic disorders, tests for determination of FDPs, D-dimers, plasminogen, t-PA, or ELISA assays for detection of fibrin monomers)

The reader may refer to subsequent chapters for a detailed discussion of vascular and platelet-related disorders (see Chapter 26), plasma clotting factor defects (see Chapter 27), interaction of systems involved in hemostasis (see Chapter 28), thrombosis and anticoagulant therapy (see Chapter 29), and laboratory methods (see Chapter 33)

CRITICAL THINKING QUESTIONS

- 25-2** Why don't laboratories routinely run the full menu of hemostatic analysis on all patients?

SUMMARY CHART

- Hemostasis is the complex process by which the body spontaneously stops bleeding and maintains blood in the fluid state within the vascular compartment.
- The vascular system, platelets, coagulation factors, and the fibrinolytic systems all play a major role in hemostasis. The kinin system and protease inhibitors play a minor, yet critical, role in hemostasis.
- Hemostasis can be divided into two stages: primary and secondary. Primary hemostasis is defined by platelet adhesion to exposed collagen within the endothelium of the vessel wall. Secondary hemostasis involves the enzymatic activation of the coagulation proteins to produce fibrin from fibrinogen, thereby stabilizing the fragile clot formed during primary hemostasis.

SUMMARY CHART—cont'd

- The principal mechanism of platelet adhesion involves (1) plasma, (2) collagen fibers, and (3) platelet membrane glycoprotein GP Ib/IX/V (the receptor for von Willebrand factor [vWF]).
- Platelets measure roughly 2 to 4 μm in diameter. Normal platelet count ranges from 150,000 to 450,000 cells/ μL . Platelets participate in hemostasis by (1) providing a negatively charged phospholipid surface for factor X and prothrombin activation; (2) release of substances that mediate vasoconstriction, platelet aggregation, coagulation (thrombin generation), and vascular repair; and (3) providing surface membrane glycoproteins to attach to other platelets via fibrinogen.
- Platelets have different and distinct zones, each with different components contributing either to their hemostatic function or their production of energy.
- The initial stage of platelet activation is as follows: platelets form pseudopods, organelles including granules and dense bodies are reorganized to the center, and contraction causes the granules to spill their contents into the open canalicular system (OCS).
- Platelet adhesion involves three components: (1) vWF, (2) glycoprotein Ib/IX/V (GP Ib/IX/V), and (3) collagen fibers.
- Platelet aggregation (platelet-to-platelet interaction) is an energy-dependent process that requires adenosine triphosphate (ATP), primarily derived from glycolysis, and involves the glycoprotein complex GP IIb/IIIa and fibrinogen.
- Four platelet-specific proteins secreted from the α granules are currently used as "markers of platelet activation." These include β -thromboglobulin, platelet factor 4, thrombospondin, and PDGF.
- Coagulation factors may be divided into three categories: substrate, cofactors, and enzymes. On the basis of physical properties, coagulation proteins may be divided into three groups: contact proteins, prothrombin proteins, and fibrinogen or thrombin-sensitive proteins.
- During thrombus formation, thrombin, plasminogen, tissue plasminogen activator, and antiplasmin are incorporated into the clot.
- Coagulation factors are designated by Roman numerals. Activation of a particular factor is designated by a lower case "a."
- Blood coagulation leading to fibrin formation can be separated into three pathways: extrinsic, intrinsic, and the common pathway.
- Fibrinolysis is the physiological process of removing unwanted fibrin deposits.
- The kinin system, important in inflammation, vascular permeability, and chemotaxis, is activated by both the coagulation and fibrinolytic systems.
- A patient's history should include (1) physical appearance, site, severity, and frequency of bleeding episodes; (2) patient and family history; (3) drug history; and (4) contributing or underlying illnesses.
- Thrombin time may be prolonged because of hypofibrinogenemia and dysfibrinogenemia, treatment with heparin, circulating fibrin degradation products (FDPs), and pathological circulating inhibitors.

CASE STUDY 25-1

A 4-year-old boy was brought to the emergency department by his parents. The boy had fallen off the monkey bars in a park and hit his thigh against one of the bars during the fall. His thigh became swollen and painful. There was a history of bleeding in the male family members. They had been labeled as "bleeders" in their family history. Coagulation studies were performed that showed a normal PT, prolonged aPTT, normal platelet count, and normal PFA-100.

QUESTIONS

1. Based on the normal PT and abnormal aPTT, which hemostatic pathway is affected in this patient?
2. What are the factors within the affected pathway?
3. Further analysis revealed that there is a low concentration of factor VIII. What condition does this indicate?
4. Does the patient's history support this diagnosis?

ANSWERS

1. The abnormal aPTT indicates an issue with at least one of the factors within the intrinsic system.
2. VIII, IX, XI, and XII
3. Hemophilia A
4. Yes, bleeding issues with hemophilia A include deep tissue bleeding and "bleeding issues" throughout life. Additionally, hemophilia A is an X-linked, recessive disorder passed from the mother, who is the carrier, to her male children. Because of inheritance patterns, male children have a 25% chance of being affected, while female children will be carriers if they inherit the affected gene.

REVIEW QUESTIONS

1. Which of the following is a minor system of hemostasis?
 - a. Vascular system
 - b. Kinin system
 - c. Coagulation
 - d. Platelets
2. The formation of fibrin strands to stabilize the platelet plug is the end product of which of the following?
 - a. Fibrinolytic system
 - b. Primary hemostasis
 - c. Kinin system
 - d. Secondary hemostasis
3. Which of the following range represents a normal platelet concentration?
 - a. 100,000–200,000/uL
 - b. 50,000–150,000/uL
 - c. 150,000–450,000/uL
 - d. 350,000–550,000/uL
4. Which of the following represents a normal platelet life span?
 - a. 1–2 days
 - b. 7–10 days
 - c. 24–35 days
 - d. 90–120 days
5. Which statement accurately describes how platelets participate in hemostasis?
 - a. Platelets provide a positively charged surface for factor activation.
 - b. Platelets release granular substrates to promote vasodilation.
 - c. Platelets provide surface glycoproteins for attachment to fibrinogen, collagen, and vWF.
 - d. Platelets provide vitamin K for coagulation.
6. Which of the following structural zones of a platelet plays a functional role in platelet activation?
 - a. Glycoproteins in the peripheral zone
 - b. Lysosomes in the organelle zone
 - c. Alpha and dense granules in the organelle zone
 - d. Dense tubular system in organelle zone
7. Shape change of a platelet and subsequent spilling of granular contents describes which of the following?
 - a. Platelet activation
 - b. Platelet aggregation
 - c. Platelet adhesion
 - d. Platelet plug stabilization
8. The linking of a platelet to collagen through GP Ib/IX/V and vWF describes which of the following?
 - a. Platelet activation
 - b. Platelet aggregation
 - c. Platelet adhesion
 - d. Platelet plug stabilization
9. The linking of a platelet to another platelet through GP IIb/IIIa and fibrinogen describes which of the following?
 - a. Platelet activation
 - b. Platelet aggregation
 - c. Platelet adhesion
 - d. Platelet plug stabilization
10. Which bleeding symptom would most likely be seen in a dysfunction of secondary hemostasis?
 - a. Hemarthrosis
 - b. Petechiae
 - c. Ecchymosis
 - d. Mucosal bleeding
11. Where are all coagulation factors produced in the body?
 - a. Bone marrow
 - b. Thymus
 - c. Spleen
 - d. Liver
12. Which factor is part of the contact protein group?
 - a. Factor II
 - b. Factor V
 - c. Factor XII
 - d. Factor IX
13. Which factor is part of the prothrombin group?
 - a. Factor II
 - b. Factor V
 - c. Factor XI
 - d. Factor XII
14. Which factor is part of the fibrinogen group?
 - a. Factor II
 - b. Factor V
 - c. Factor XI
 - d. Factor XII
15. What initiates the extrinsic pathway?
 - a. Release of tissue factor from injured vessel wall
 - b. Contact factors activation
 - c. Activation of factor X
 - d. Fibrinogen transformation into fibrin
16. What is thrombin's role in the common pathway?
 - a. Binding to platelet membrane receptors
 - b. Amplification of factors V
 - c. Positive feedback to amplify formation of thrombin from prothrombin
 - d. Promoting secretion of serotonin
17. Which enzyme is responsible for lysis of the fibrin clot?
 - a. Fibrinogen
 - b. Thrombin
 - c. Plasmin
 - d. Collagen

REVIEW QUESTIONS—cont'd

18. Kallikrein is involved in which system of hemostasis?
 - a. Prothrombin group
 - b. Fibrinogen group
 - c. Thrombin group
 - d. Contact activation group
19. A deficiency of factor VIII would be detected with which analysis?
 - a. PT
 - b. aPTT
 - c. PFA
 - d. D-dimer
20. The thrombin time is the best measure for which of the following?
 - a. Dysfunction within the extrinsic pathway
 - b. Dysfunction within the intrinsic pathway
 - c. A fibrinogen deficiency
 - d. DIC

See answers at the back of this book.

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Disorders of Primary Hemostasis

Quantitative and Qualitative Platelet Disorders and Vascular Disorders

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CHAPTER OUTLINE

Laboratory Evaluation of Disorders of Primary Hemostasis

Quantitative Platelet Disorders:

Thrombocytopenia

Deficient Platelet Production

Abnormal Distribution of Platelets

Increased Destruction of Platelets

Quantitative Platelet Disorders:

Thrombocytosis

Primary Thrombocytosis

Reactive Thrombocytosis

Qualitative Platelet Disorders

Congenital Disorders of Platelet

Function

Acquired Qualitative Platelet Disorders

Vascular Disorders

Primary Purpura

Secondary Purpura

Vascular and Connective Tissue Disorders

Summary Chart

Case Study 26-1

Case Study 26-2

Case Study 26-3

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter the learner should be able to:

- 26-1 Describe the laboratory tests that may be utilized in the evaluation of quantitative and qualitative platelet disorders.
- 26-2 Describe the pathophysiologic processes that cause thrombocytopenia and their associated disorders.
- 26-3 Define immune-mediated thrombocytopenia (ITP).
- 26-4 Name conditions that are associated with autoimmune and alloimmune thrombocytopenia.
- 26-5 Compare ITP and thrombotic thrombocytopenic purpura (TTP).
- 26-6 Contrast thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS).
- 26-7 Indicate the laboratory studies used for diagnosis of storage pool and platelet release defects.
- 26-8 Differentiate between reactive and primary thrombocytosis, including expected hemostatic problems for each.
- 26-9 Compare and contrast Bernard-Soulier syndrome from Glanzmann's thrombasthenia.
- 26-10 Differentiate among von Willebrand disease, Bernard-Soulier syndrome, and hemophilia A, including laboratory tests and results utilized to diagnose each.
- 26-11 Identify inherited and acquired vascular defect disorders.

Disorders of primary hemostasis include abnormalities that clinically result in bleeding. This is due to defects in the formation of an adequate platelet plug on an injured blood vessel wall. A platelet plug may not form adequately because of quantitative abnormalities (abnormal platelet concentrations), qualitative platelet disorders (abnormalities in platelet function), or abnormalities of the blood vessel wall. These disorders can be inherited or acquired. Patients typically present differently clinically depending on whether their platelet disorder is caused by an inherited or acquired disorder.

In inherited primary hemostatic disorders, the patient usually has a history of childhood bleeding, such as gingival bleeding or a history of easy or spontaneous bruising. In contrast, acquired disorders usually will not have a history of childhood bleeding and present with signs of bleeding beginning in

adulthood. It is always important to rule out a history of drugs or other underlying diseases, which may cause the bleeding episode. This is where laboratory evaluation can prove invaluable for patient diagnosis.

Regardless of the disorder at the cause, primary hemostasis abnormalities typically result in clinical manifestations limited to skin, such as ecchymosis (petechiae or purpura) or mucosal bleeding, which can include epistaxis, gingival bleeding, gastrointestinal bleeding, menorrhagia, or hematuria. Spontaneous hemarthrosis and hematomas of deep structures are typical features of defects in secondary hemostasis (coagulation protein deficiency states), and usually are not seen in platelet disorders or vascular defects (see Chapter 27).

The diagnosis of any hemostatic disorder is made by the systematic evaluation of information obtained in the history

and physical examination, along with the appropriate laboratory testing. Bleeding disorders present differently, depending on the hemostatic defect causing the disorder. It is important to establish whether bleeding in an individual is due to a local blood vessel injury or caused by a systemic hemostatic defect, especially in the bleeding surgical patient.

To understand platelet quantitative and qualitative disorders, it is important to review the normal platelet physiology and function, which is provided in Chapter 25. This chapter reviews the etiology, pathophysiology, clinical manifestations, and laboratory tests used to identify the various quantitative and qualitative platelet disorders as well as the numerous vascular disorders.

Laboratory Evaluation of Disorders of Primary Hemostasis

Although many laboratories differ in their approach to bleeding disorders, a general profile of laboratory tests is usually established. This profile can often be used as a means of classifying bleeding disorders, as shown in Table 26-1. The laboratory tests that aid in the evaluation of disorders of primary hemostasis are listed in Box 26-1.

The automated platelet count is one of the most important initial tests to evaluate a bleeding tendency because acquired quantitative platelet disorders are the most common disorders of primary hemostasis. Visual inspection of the peripheral smear can be used to confirm the automated platelet count and reveal platelet morphology. The peripheral smear should be carefully inspected for evidence of large or **dysplastic platelets**. Direct inspection should also assess for platelet satellitism or platelet clumping, which may cause a falsely low automated platelet count and is termed **pseudothrombocytopenia**. When pseudothrombocytopenia is suspected, a "true" automated count can often be obtained by performing an automated platelet count in alternative anticoagulants such as citrate or heparin or platelet estimate from the peripheral blood smear. This phenomenon does not result in clinical bleeding but may be confused with other thrombocytopenia syndromes, resulting in unnecessary laboratory evaluations and inappropriate treatments.¹ It is usually a result of ethylene diamine tetraacetic acid (EDTA)-dependent cold agglutinins.^{1,2} These antibodies have been shown to be IgG antibodies that are

BOX 26-1 Laboratory Tests to Assess Disorders of Primary Hemostasis

Platelet count
Peripheral blood smear
PFA-100
Von Willebrand studies
• FVIII:C
• vWF antigen
• vWF activity
Platelet antibody testing
Flow cytometry
• Platelet glycoprotein analysis
• Platelet-associated IgG
Platelet aggregation studies
Lumiaggregometry
Bone marrow aspiration and biopsy

FVIII:C = factor VIII:C; vWF = von Willebrand factor; PFA = platelet function analyzer.

EDTA-dependent and bind to the platelet membrane glycoprotein IIb/IIIa complex.²

The bleeding time has been utilized in the past as an *in vivo* screening test to evaluate bleeding caused by platelet and vascular disorders by several methods. However, despite attempts to standardize the performance of this test, the bleeding time is not reliable for diagnosis of a primary hemostatic defect or predictive of bleeding risk in individuals.³ Newer *in vitro* automated testing methods have replaced this test in clinical practice. The automated platelet function analyzer (PFA-100) has largely made the bleeding time obsolete because of its greater precision, accuracy, and reliability.⁴ As this is an *in vitro* test, it does not assess for abnormalities of blood vessels in primary hemostasis. A sample of the patient's blood is drawn into a citrated tube and the citrated blood is then run through a cartridge with either ADP and collagen or epinephrine and collagen. The cartridge has a small aperture, and the instrument measures the amount of time required for closure of the aperture by a small platelet plug. In some instances, the collagen/epinephrine cartridge is run first, and if abnormal, the collagen/ADP cartridge is run. However, often laboratories run both cartridges as a platelet function screen. Both the ADP and epinephrine closure times are prolonged in patients with von Willebrand disease, inherited qualitative platelet disorders, and quantitative platelet disorders (thrombocytopenia when the platelet count is less than 100,000/ μ L).¹ Aspirin and other platelet inhibitors have been shown to have variable effects on the PFA-100 closure times. In addition, lower hematocrits (<35%) and increased erythrocyte sedimentation rates will also affect the PFA-100 closure times, usually prolonging them.

Because von Willebrand disease mimics the bleeding diathesis of platelet disorders, assays to exclude this diagnosis are an integral part of the evaluation. To determine the presence or absence of von Willebrand disease, factor VIII coagulant

TABLE 26-1 Classification of Bleeding Disorders by Screening Tests

Test	Vascular Disorder	Quantitative Platelet Disorder	Qualitative Platelet Disorder
Platelet count	N	AbN	N
PT	N	N	N
APTT	N	N	N
PFA-100 Assay	AbN	AbN	AbN

N = normal; AbN = abnormal; PT = Prothrombin time; APTT = activated partial thromboplastin time; PFA = platelet function analyzer.

activity, von Willebrand antigen (vWF:Ag), von Willebrand activity by ristocetin-induced platelet aggregation, and von Willebrand multimers must be assayed together. Further discussion of these tests is provided later in this chapter in the section on von Willebrand disease.

Platelet antibody testing determines the amount of immunoglobulin G (IgG) bound on the platelet surface by various immunological methodologies. Flow cytometry can be used to measure platelet-bound IgG and platelet surface glycoproteins. Increased amounts of platelet-associated IgG are often found in immune-mediated thrombocytopenias, but this finding is usually not specific enough to establish a diagnosis of an immune origin except in rare instances such as heparin-induced thrombocytopenia.

Platelet aggregation by light transmission aggregometry assesses platelet function by measuring the response of the platelet to various stimuli such as epinephrine, adenosine diphosphate (ADP), collagen, thrombin, and ristocetin. The procedure for performing platelet aggregation is described in Chapter 33. Abnormalities of the platelet surface and release defects can be identified with this test.

ADVANCED CONTENT

Drugs and disorders such as hepatic or renal disease can interfere with platelet function and make interpretation of this test difficult. Lumiaggregation, which measures ATP release from platelet dense granules in concert with aggregation by luminescence from a firefly luciferase reaction dependent on the presence of ATP, can be useful to specifically measure platelet release. Platelet function disorders may be challenging for diagnostic laboratories to evaluate with resource limitations influencing which tests to provide.⁵ Newer automated methodologies to measure platelet function and global hemostasis include thromboelastography (TEG) and rotational thromboelastography (ROTEM). These tests may be performed as point-of-care test during surgery to aid in identification of primary and secondary hemostatic abnormalities and guide administration of corrective therapies. The "Verify Now" aspirin assay system may be used to measure platelet responsiveness to aspirin, and the Verify Now system may be used to measure ADP receptor blockade by P2Y₁₂ platelet inhibitors (clopidogrel, ticagrelor, and prasugrel).⁶

Bone marrow aspiration and biopsy may be useful in determining the etiology of quantitative platelet disorders. The bone marrow specimen can assess the adequacy of megakaryocytes, overall cellularity, and to identify infiltrative processes such as myelodysplastic syndromes and malignancy or fibrosis. (Refer to Chapter 20.)

Quantitative Platelet Disorders Thrombocytopenia

Platelets must be present in adequate numbers to maintain normal hemostasis. Platelet production remains relatively constant

for an individual over time. The body senses the total platelet mass, related both to number and mean volume of platelets, and regulates this tightly. **Thrombopoietin (TPO)**, a growth factor produced by the liver and to a lesser degree by the spleen, is responsible for this regulation. When the platelet mass is normal, TPO is cleared from plasma by TPO receptors on platelets and megakaryocytes. With thrombocytopenia, the overall clearance is low and the plasma concentration of TPO increases to boost megakaryocyte and platelet production. The average platelet count ranges from 150 to $400 \times 10^9/L$ of whole blood. **Thrombocytopenia** is defined as a platelet count under the lower limit of normal, although clinical signs and symptoms of thrombocytopenia typically are not manifested until the platelet count falls below $100 \times 10^9/L$ and, commonly, not until the platelet count falls below $50 \times 10^9/L$. Overt spontaneous hemorrhage is not usually seen until the platelet count falls to less than $20 \times 10^9/L$. Platelet counts less than 10,000 may result in life-threatening hemorrhage and may require emergency platelet transfusions or other treatments. Quantitative platelet disorders are the most commonly encountered group of platelet abnormalities and can be simply divided into two categories, thrombocytopenia and thrombocytosis. Thrombocytopenia results from three distinct mechanisms: deficient platelet production, abnormal platelet distribution (splenic sequestration), and increased platelet destruction (Table 26-2). Qualitative platelet defects may coexist with quantitative platelet defects to increase bleeding risk.

CRITICAL THINKING QUESTION

26-1 Why do quantitative platelet disorders place an individual at risk for bleeding issues?

See answers to all Critical Thinking Questions at the back of this book.

Deficient Platelet Production

Impaired platelet production resulting in thrombocytopenia may be caused by many disorders. These disorders produce megakaryocytic **hypoplasia** often with erythroid and granulocytic hypoplasia, resulting in pancytopenia. These platelet disorders can occur spontaneously (aplastic anemia) or as a result of injury to the bone marrow (radiation or chemotherapy). Replacement of marrow hematopoietic tissue from infiltrative processes such as myelofibrosis, leukemia, Hodgkin's and non-Hodgkin's lymphoma, and metastatic cancer result in pancytopenia (anemia, thrombocytopenia, and leukopenia). The blood smear is characterized by nucleated red blood cells, teardrop-shaped cells (dacrocytes), and immature granulocytes. These characteristic findings on the peripheral smear are often referred to as a **myelophthitic picture** and should make one highly suspicious of an infiltrative process within the bone marrow. Bone marrow aspiration and biopsy are indicated to confirm a diagnosis of marrow aplasia or infiltration when these findings are present.

Thrombocytopenia Due to Ineffective Hematopoiesis

Ineffective hematopoiesis is associated with normal or increased marrow cellularity but peripheral blood cytopenia. Megaloblastic anemia associated with vitamin B₁₂ or folic acid deficiency is commonly associated with thrombocytopenia as

TABLE 26-2 Classification of Disorders Causing Thrombocytopenia

Classification	Type	Specific Disorders
Deficient Platelet Production	Myelophthitic (marrow infiltrative processes)	<ul style="list-style-type: none"> • Leukemia • Lymphoma • Multiple myeloma • Metastatic carcinoma • Myelofibrosis
	Aplasia	<ul style="list-style-type: none"> • Aplastic anemia (Fanconi's anemia) • Amegakaryocytic thrombocytopenia • Drug effect (chemotherapy) • Radiation therapy
	Ineffective erythropoiesis	<ul style="list-style-type: none"> • Pernicious anemia (vitamin B₁₂ deficiency) • Folic acid deficiency • Alcohol ingestion • Myelodysplasia • Paroxysmal nocturnal hemoglobinuria
	Congenital disorders	<ul style="list-style-type: none"> • May–Hegglin syndrome • Thrombocytopenia with absent radii (TAR) syndrome • Bernard–Soulier syndrome • Familial thrombocytopenia
Abnormal Platelet Distribution		<ul style="list-style-type: none"> • Hypersplenism (splenomegaly) • Hemangiomas (Kasabach–Merritt syndrome)
Increased Platelet Destruction	Immune (primary)	<ul style="list-style-type: none"> • Idiopathic thrombocytopenic purpura (ITP) • Post-transfusion purpura • Neonatal isoimmune purpura • Drug-induced thrombocytopenia • Vaccine-induced thrombocytopenia • Heparin-induced thrombocytopenia and thrombosis
	Immune (secondary)	<ul style="list-style-type: none"> • Lymphoproliferative disorders • Systemic lupus erythematosus/collagen vascular disorders • Viral infection (mononucleosis, measles, HIV)
	Microangiopathic thrombocytopenia	<ul style="list-style-type: none"> • Thrombotic thrombocytopenic purpura (TTP) • Hemolytic uremic syndrome (HUS) • Disseminated intravascular coagulation (DIC)
	Pregnancy-associated thrombocytopenia	<ul style="list-style-type: none"> • Gestational thrombocytopenia • Preeclampsia–eclampsia and HELLP syndrome

HIV = human immunodeficiency virus; HELLP = hemolysis elevated liver enzymes and low platelet count.

a result of impaired DNA synthesis. Serum lactate dehydrogenase (LD) levels are elevated as a result of intramedullary death of hematopoietic progenitors. Thrombocytopenia is generally mild. Platelet life span has been reported as being normal to only slightly decreased. Myelodysplastic syndromes may simulate vitamin deficiencies but do not respond to vitamin replacement. Chromosomal abnormalities are often present. Paroxysmal nocturnal hemoglobinuria, a rare disorder with increased cellular sensitivity to complement, is also associated with cytopenias resulting from intramedullary cellular destruction. These disorders are discussed in detail in Chapters 8, 13, and 20.

Alcohol has a direct toxic effect on the marrow, thereby producing thrombocytopenia in the absence of a folic acid or vitamin B₁₂ deficiency. Mild thrombocytopenia and acquired platelet function defects appear to improve after the use of alcohol is stopped.

Congenital Thrombocytopenia

A number of inherited disorders produce thrombocytopenia; however, all are rare.⁷ Patients with thrombocytopenia with absent radius (TAR) syndrome have absent radii in addition to the thrombocytopenia. Eczema and immunodeficiency are associated with Wiskott–Aldrich syndrome, an X-linked disorder only occurring in males exhibiting very small platelets. Bernard–Soulier syndrome and May–Hegglin anomaly have abnormally large platelets but do not have associated skin or skeletal defects. However, individuals with May–Hegglin anomaly may develop hearing and kidney problems. Familial thrombocytopenia mimics immune thrombocytopenic purpura clinically but affects other family members, in contrast to immune thrombocytopenic purpura. Several different genetic mutations have been discovered to cause familial thrombocytopenia. Some of these disorders also exhibit a qualitative

abnormality in platelet function and are discussed under qualitative platelet disorders later in this chapter. A list of the more common congenital disorders and their associated abnormalities is provided in Table 26-3.

Abnormal Distribution of Platelets

Normally the spleen pools approximately one-third of the platelets produced by the marrow. When the spleen enlarges, more platelets can be sequestered within, leading to thrombocytopenia. Many conditions, including liver cirrhosis, hematologic malignancies, and portal vein thrombosis, can cause hypersplenism. Typically, the platelet count remains greater than $50 \times 10^9/L$ in patients with hypersplenism. Kasabach-Merritt syndrome, a rare disorder, results in platelet sequestration in giant hemangiomas.

Increased Destruction of Platelets

Immune-Mediated Thrombocytopenias

This group of thrombocytopenias all have an immune-mediated mechanism by which there is increased platelet destruction that can be primary (idiopathic) or secondary to an underlying disease (see Table 26-2).

Immune Thrombocytopenic Purpura

Immune thrombocytopenic purpura (ITP) is one of the most common disorders causing severe isolated thrombocytopenia and is caused by an autoantibody to the patient's platelets. There is not a specific test that readily confirms the diagnosis of ITP, so it is typically a diagnosis of exclusion.⁸ ITP can present in children and adults; however, there are important differences between the two groups when comparing the long-term prognosis (Table 26-4).

TABLE 26-3 Congenital Disorders Associated With Decreased Platelet Production

Disorder	Associated Abnormalities
Alport's syndrome	Giant platelets Thrombocytopenia Deafness Nephritis
Cnédiak-Higashi syndrome	Partial oculocutaneous albinism Increased susceptibility to pyogenic infections Storage pool defect of dense granules
Hermansky-Pudlak syndrome	Platelet deficiency of nonmetabolic ADP Oculocutaneous albinism
May-Hegglin anomaly	Thrombocytopenia Giant platelets Döhle bodies
TAR syndrome	Multiple skeletal and cardiac abnormalities Storage pool defect
Wiskott-Aldrich syndrome	Disorders of dense granules Recurrent pyogenic infections Eczema Thrombocytopenia

TABLE 26-4 Comparison of Acute and Chronic ITP

Characteristics	Acute	Chronic
Age at onset	Child	Adult
Previous infection	Yes	No
Platelet count	Usually $\leq 20,000$	Usually 30–50,000
Bleeding episode	Abrupt	Slow
Duration of thrombocytopenia	Transient	Prolonged
Spontaneous remission	Yes	No

Childhood ITP Young children may present with an immune thrombocytopenia that typically develops acutely with a 1- to 2-week duration, usually with bruising or petechiae.⁹ It may develop following a viral infection but often there is no clear cause. Serious bleeding is uncommon. Most children present with initial platelet counts of less than $20 \times 10^9/L$. Bone marrow aspiration and biopsy is often performed to exclude the diagnosis of acute leukemia. This disorder is usually self-limited, lasting about 6 to 8 weeks. Spontaneous remissions, with or without therapy, occur in the majority of these patients. However, immunoglobulin G, anti-D immunoglobulin, and corticosteroids may be used to decrease the period of thrombocytopenia and stop bleeding. A minority of children with a more chronic form are treated like the more common adult form of ITP. Splenectomy is usually avoided because of the infectious complications of splenectomy in young children.

Adult ITP In adults, ITP commonly presents in the 20- to 50-year-old group as a chronic disease process.¹⁰ Occasionally, patients will have an acute immune thrombocytopenia after a viral illness, vaccinations, or exposure to drugs, but this is uncommon. In most cases of adult immune thrombocytopenia, there is usually not a recent history of drug exposure or infectious illness that can be related to the onset of thrombocytopenia. Platelet counts are typically less than $30 \times 10^9/L$ in patients who present with bleeding manifestations. Clinically, patients present with mucosal bleeding typical of a primary hemostatic defect, such as menorrhagia, epistaxis, easy bruisability, or petechiae (Figs. 26-1 and 26-2). Some patients are diagnosed while still asymptomatic based on a low platelet count on a routine complete blood count obtained for other reasons. These patients usually have a platelet count greater than $50 \times 10^9/L$. Adult ITP does not usually remit spontaneously.

Common Clinical Findings

The bone marrow in adult ITP is characterized by increased or normal numbers of megakaryocytes (Fig. 26-3). Platelet life span is shortened and circulating platelets are morphologically large on the smear, reflecting the early release from the marrow in response to the peripheral destruction. There are also changes in the splenic microcirculation that cause a reduction of platelet transit time in the spleen and increased destruction of the antibody-coated platelets. There may also be diminished platelet production that limits the marrow compensatory response to the platelet destruction.



FIGURE 26-1 Oral cavity of a patient with idiopathic thrombocytopenic purpura (ITP).

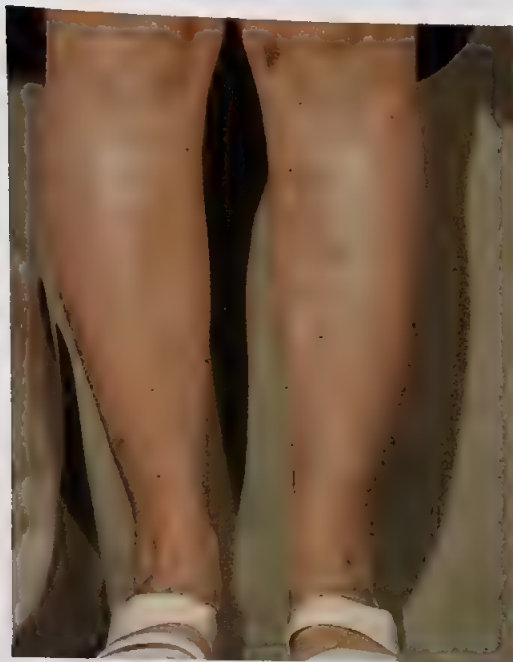


FIGURE 26-2 Petechial bleeding of the lower extremities in a patient with ITP.

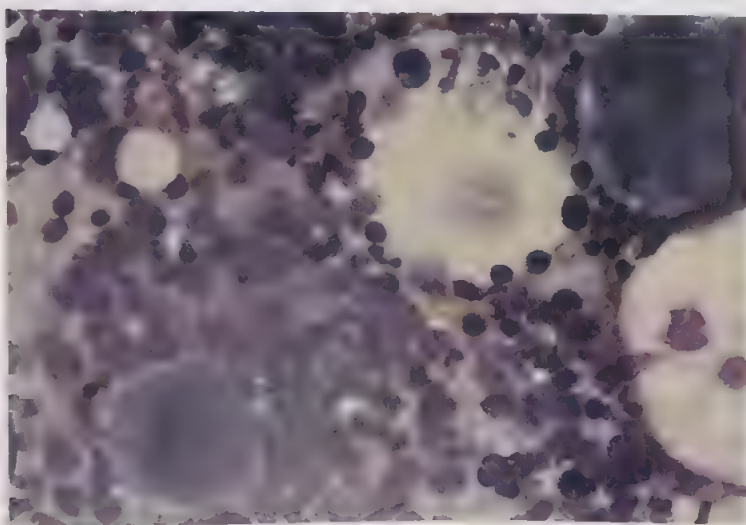


FIGURE 26-3 ITP, bone marrow aspirate. Note the increased number of megakaryocytes with normal cellularity (M/E 3:1).

Because ITP is a diagnosis of exclusion, other causes of thrombocytopenia must be considered and eliminated. Drugs that may cause thrombocytopenia must be stopped and consideration given to a bone marrow aspiration and biopsy to exclude a primary bone marrow disorder if other cytopenias are present.

Laboratory Testing and Results

Because ITP is a diagnosis of exclusion, there are no definitive confirmatory tests for this disorder. Platelet count less than 100,000/ μ L, and large platelets might be seen on a peripheral blood smear. Testing for hepatitis C and human immunodeficiency virus (HIV) is generally recommended, as these viral infections can mimic ITP, but there are specific antiviral treatments available to control these diseases. Antiplatelet antibody tests, which measure IgG bound to the platelet surface, have been used to confirm the diagnosis of ITP; however, these tests are not specific and are not recommended for diagnosis.

Treatment

A decision to initiate treatment is based on platelet count and bleeding symptoms. Treatment is recommended when the platelet count is less than 30,000/ μ L; however, due to the variability in bleeding risk in different individuals and the potential for adverse effects of therapy, some patients at that platelet threshold may be closely monitored without initiating treatment. Patients with ITP are initially treated with corticosteroids to rapidly increase the platelet count and improve hemostasis. Corticosteroids are thought to reduce autoantibody production and to suppress splenic sequestration of moderately sensitized platelets, thereby increasing the platelet life span and ameliorating the thrombocytopenia. Choices for corticosteroid therapy include a short course of high-dose daily prednisone for up to 6 weeks, or a 4-day course of high-dose dexamethasone, which might need to be repeated to achieve a response.

Approximately 70% to 90% of patients initially respond favorably to steroids. However, sustained responses are usually seen in only about 25%. If a patient is intolerant of corticosteroids or does not achieve a satisfactory response, intravenous immunoglobulin G may be used acutely to increase the platelet count and stop bleeding. Most patients also usually respond to intravenous immunoglobulin G. The response is usually transient and must be repeated to sustain a remission. There does not appear to be a more rapid increase of the platelet count with both of these agents compared with either alone. Anti-D immunoglobulin can be used in some individuals with ITP who are Rh-positive. Its mechanism of action is probably similar to that of immunoglobulin G, but it has the benefits of being a short infusion over 5 minutes and not requiring the fluid volume necessary to administer immunoglobulin G. There is often a mild hemolysis of red blood cells associated with the administration of anti-D globulin; therefore, the dosage should be adjusted according to the hemoglobin level at the time of treatment. The time to response with any of these initial treatments varies with each individual, although most responses are seen within a week or two of initiation of therapy. Thrombocytopenia usually recurs when these treatments are stopped.

Splenectomy has been the preferred long-term treatment of choice for ITP in relapse after an initial response to steroid therapy or immunoglobulin G. Significant improvement following a splenectomy is obtained in 50% to 80% of patients. The benefit of splenectomy results from the removal of the organ responsible for the autoantibody production and the sequestration of moderately sensitized platelets. However, its use as a second line therapy has largely been supplanted by other less invasive treatments which are usually considered first.

Rituximab, an anti-CD 20 monoclonal antibody, has become a preferred treatment for individuals refractory to primary treatment as it does not have the surgical and infectious risks of splenectomy. Thrombopoietin (TPO) mimetics are often used as second line treatments after demonstrating efficacy in randomized clinical trials in this setting. Romiplostim is administered weekly as a subcutaneous injection. Eltrombopag and avatrombopag are administered orally daily. The choice between these therapies is usually based on patient preferences and other factors. In patients who are actively bleeding, antifibrinolytic agents such as epsilon (ε)-aminocaproic acid (EACA) or tranexamic acid can be used to help control bleeding until the platelet count can be corrected. These agents are safe to use either before or after splenectomy and are most effective at sites of high fibrinolytic activity (i.e., urinary tract, nose, and mouth). Platelet transfusions are often not effective because transfused platelets are usually rapidly destroyed; however, in some patients a platelet response can occur and bleeding might improve.¹¹

ADVANCED CONTENT

Rituximab can be used for initial therapy in combination with corticosteroids and immunomodulatory agents, such as cyclosporine A, to try to achieve better, longer lasting initial responses. Many other agents have been utilized in patients who do not respond to splenectomy or who relapse after splenectomy or rituximab. Unfortunately, the studies of efficacy of these treatments have involved relatively small numbers of patients. Some of the agents that have been attempted include danazol; dapsone; colchicine; vitamin C; chemotherapeutic agents such as vincristine, cyclophosphamide, or combination chemotherapy; and immunosuppressive therapy such as azathioprine, mycophenolate mofetil, or cyclosporine A. The next most effective agent when there has been failure of response to splenectomy, Rituximab or a TPO mimetic, has not been clearly defined. The decision about which treatment modality to utilize varies according to the treating physician.

Post-Transfusion Purpura (PTP)

In this disorder, sudden onset of thrombocytopenia occurs within 2 weeks after transfusion of blood products containing platelets¹² (Fig. 26-4). The majority of cases are a result of an alloantibody directed against the platelet antigen PI^A , also referred to as HPA-1a.¹⁴ The PI^A antigen is found in approximately 97% of the



FIGURE 26-4 Post-transfusion purpura (PTP)

normal population; the 3% of people who lack the PI^A (HPA-1a) antigen on their platelets are considered at risk for developing PTP. It is believed that **post-transfusion purpura (PTP)** results from an anamnestic immune response from prior exposure to the antigen. Most reported cases have been in middle-aged women who have had children. It is believed that primary immunization occurs during pregnancy, when PI^A -positive fetal platelets sensitize a PI^A -negative mother (see the following discussion of isoimmune neonatal thrombocytopenia). Three mechanisms to explain this phenomenon have been proposed: 1) immune complexes of anti-HPA-1a and transfused soluble HPA-1a bind to autologous platelets, leading to immune clearance; 2) transfused soluble HPA-1a binds to autologous platelets and leads to clearance by HPA-1a antibodies; and 3) HPA-1a autoantibodies are generated along with the HPA-1a alloantibodies and all are responsible for the destruction of autologous platelets.

Treatment with platelet transfusions is usually not effective as the alloantibodies destroy even PI^A -negative platelets. Corticosteroids are often used. Plasmapheresis without plasma exchange and intravenous IgG infusions have each been an effective means of treating the hemorrhagic complications associated with PTP. In a number of cases, patients have had repeated episodes of PTP after reexposure to PI^A -positive blood, but some do not. PI^A -negative blood is indicated for all subsequent transfusions when possible because patients are considered at risk for recurrence of PTP with subsequent transfusions.

ADVANCED CONTENT

Human platelet antigens (HPAs) have been described on six different platelet glycoproteins in which 41 HPAs have been expressed. Rare reports of cases of PTP have occurred in association with antibodies to these HPAs in the sera of patients.¹² HPA testing is highly specialized and is usually performed in reference laboratories. This testing involves platelet antibody testing and HPA genotyping. Direct and indirect laboratory tests have been developed to increase

specificity and sensitivity in the detection of platelet antibodies against glycoprotein specific HPA antigens. Direct tests detect antibodies attached to the patient's platelets but are limited if there is severe thrombocytopenia. Indirect tests identify unbound platelet antibodies in serum. These tests employ some of the following techniques: modified antigen capture ELISA (MACE), platelet antibody and bead array (PABA), and monoclonal antibody specific immunization of platelets (MAIPA). HPA typing of DNA by polymerase chain reaction or next generation sequencing may be used to confirm antibody specificity and guide the care for future transfusion therapy. In some cases of PTP, iso-sensitization to HLA antigens found on platelets occurs, as well as the appearance of platelet-specific antigens other than PI^A1 , making serological typing difficult.

Isoimmune Neonatal Thrombocytopenia

Similar to the pathogenesis of erythroblastosis fetalis, **isoimmune neonatal thrombocytopenia** results from immunization of the mother by fetal platelet antigens and placental transfer of maternal antibody. Isoimmune neonatal thrombocytopenia is most often caused by maternal alloantibodies to the PI^A1 (HPA-1a) antigen.¹³

▶ ADVANCED CONTENT

Similar to post-transfusion purpura, isoimmune neonatal thrombocytopenia has been reported only rarely with other platelet antigens such as PI^A2 , Bak^a , Bak^b , Br^a , and Br^b . It is an uncommon disorder, generally affecting the first-born child. Based on gene frequency of the PI^A1 (HPA-1a) antigen in fathers, there is a high probability that a PI^A1 -negative mother will have a PI^A1 -positive child. Once isoimmune neonatal thrombocytopenia has developed, there appears to be an increased risk of the next child being affected, because most fathers are homozygous for PI^A1 .

A large percentage of PI^A1 -negative mothers who give birth to an affected child are phenotype-positive for the HLA-B8 antigen. It has been suggested that the HLA-B8 antigen serves to protect from immunization, which accounts for the relatively low incidence of isoimmune neonatal thrombocytopenia, despite the frequency of the PI^A1 antigen and the chance for maternal sensitization. The relationship of ABO incompatibility to symptomatic isoimmune neonatal thrombocytopenia is unclear.

Infants who develop isoimmune neonatal thrombocytopenia appear normal at birth but within hours develop scattered petechiae and purpuric hemorrhages, with platelet counts under $30 \times 10^9/L$. Intracranial hemorrhage is the primary cause of mortality in these infants. Characteristically, in this disorder the platelet count begins to decrease shortly after birth with low levels reached several hours later.

Therapy is aimed at preventing intracranial hemorrhage and keeping platelet counts at hemostatically safe levels.

Caesarean delivery is usually performed to prevent intracranial hemorrhage from birth trauma when the disorder is suspected before delivery. Corticosteroids or intravenous IgG may be used prior to delivery. Postnatal treatment is not necessary if the infant is asymptomatic and the platelet count remains above $30 \times 10^9/L$. When the infant manifests clinical signs of bleeding and the platelet count falls below $10 \times 10^9/L$, compatible platelet transfusions utilizing maternal platelets or PI^A1 -negative donor platelets is the preferred treatment.

Drug-Induced Immune Thrombocytopenia

Numerous medications may cause thrombocytopenia by various mechanisms.^{14,15} There may be nonimmune suppression of thrombopoiesis and induction of apoptosis. Different immune mechanisms may cause drug-induced ITP. A list of causes of drug-induced thrombocytopenia is provided in Box 26-2. Quinine, which is used to treat malaria and is found in beverages such as tonic water, was one of the first drugs noted to cause thrombocytopenia. To cause this problem, the drug appears to bind to platelet glycoproteins and allows platelet reactive antibodies to bind but only when the drug is present (drug-dependent antibody), leading to clearance of antibody-coated platelets in the spleen.

Penicillins elicit an antibody response only when complexed with a larger carrier molecule on the surface of platelets (hapten-induced antibodies), leading to platelet destruction in the spleen. Tirofiban and eptifibatide bind to platelet glycoproteins IIb/IIIa (GP IIa/IIb) and are used for percutaneous coronary interventions to prevent thrombosis. The drugs appear to create a new epitope on GP IIb/IIIa, which becomes a target for natural antibodies or antibodies that occurred from previous exposure to these drugs. Antibody-coated platelets are cleared by the spleen but also may become activated and produce thrombosis. Monoclonal antibodies can cause thrombocytopenia due to natural antibodies recognizing the murine component of the drug (abciximab) or circulating immune complex deposition causing complement-induced platelet lysis or direct binding of these immune complexes (rituximab) to its CD20 target antigen. Another

BOX 26-2 Causes of Drug-Induced Thrombocytopenia

Nonimmune direct suppression of platelet function (i.e., chemotherapy)

Immune-mediated platelet destruction

- Drug-dependent antibodies (i.e., quinine)
- Hapten-induced antibodies (i.e., penicillins)
- Fiban dependent antibodies (i.e., tirofiban, eptifibatide)
- Fab-binding antibodies (i.e., abciximab, rituximab)
- Drug-induced autoantibody formation (i.e., sulfonamides)
- Immune complex formation (i.e., heparins)
- Immune-mediated suppression of platelet production (i.e., quinine, eptifibatide)

immune complex-mediated thrombocytopenia occurs related to unfractionated and low molecular weight heparins (see separate discussion later). Immune-mediated suppression of platelet production related to direct antibody binding to megakaryocytes may occur with eptifibatide and quinine, in addition to platelet destruction.

The list of drugs that have been implicated to cause immune drug purpura is rather extensive. The drugs most frequently cited are quinine, quinidine, trimethoprim/sulfa, and vancomycin. However, immunological testing to confirm the presence of platelet-bound antibodies is lacking in many of those reports. The presence of drug-dependent antibodies can confirm the diagnosis, but the lack of standardization and validation of the various immunoassays for various drugs can be problematic. A negative immunoassay does not rule out drug-induced immune thrombocytopenia. A thorough drug history that includes prescription medications, herbal products, and beverages, as well as certain natural foods (cow's milk, walnuts, cranberry juice, and sesame seeds) is important to consider this possibility. When possible, any suspect drug should be stopped in a thrombocytopenic patient. Drug-induced immune purpura appears to occur more frequently in the elderly population as a result of the increased usage of medications; however, cases have been reported in children and young adults. Profound thrombocytopenia occurs with platelet counts less than $20 \times 10^9/L$ and bleeding is common. Purpura occurs approximately 7 days after initial use of the drug but may occur within 3 to 5 days owing to an anamnestic response on reexposure to the drug. The disorder is generally self-limiting because the platelet count rapidly returns to normal once the drug has been removed from circulation. The incidence of drug-induced immune thrombocytopenic purpura is difficult to estimate for various suspect medications as causality is often not clearly established in many of the published reports. Readministration of a drug suspected to cause purpura confirms cause but should be avoided as serious bleeding may occur with rechallenge.

Heparin-Induced Thrombocytopenia and Thrombosis

Heparin therapy is associated with the development of two distinct types of thrombocytopenia. One type develops early in treatment and is benign. The platelet count rarely falls below $100 \times 10^9/L$, and there are no resultant bleeding or thrombotic complications. The second type is associated with severe thrombocytopenia and, paradoxically, thrombotic episodes instead of hemorrhagic complications (see Chapter 29). Initially, it may be difficult to distinguish between these two types of thrombocytopenia based on laboratory values alone.

In the second type of thrombocytopenia, platelet counts as low as $20 \times 10^9/L$ occur in association with arterial and venous thrombosis; this type has been termed heparin-induced thrombocytopenia and thrombosis syndrome (HITTS).¹⁶ The actual incidence of this syndrome is not well defined, estimated to be anywhere from <0.1% to 7% depending on the patient population and heparin exposure.¹⁷ HITTS typically develops 4 to 7 days after initial exposure to heparin; however, in individuals who have been previously exposed to heparin, it can

develop within 1 to 3 days after reexposure. Patients with myocardial infarction and cardiogenic shock or those who have undergone major vascular surgery may be particularly susceptible. Venous or arterial thrombosis results in an incidence of morbidity and mortality of up to 20% for patients who develop HITTS.

Pathologically, this syndrome develops secondary to antibody produced to platelet factor 4-heparin complex with immune complex Fc receptor-induced activation of platelets.

However, the antibodies are of the IgG isotype, not IgM, which should occur with a primary immune response. In heparin-naïve patients, the anti-PF4/heparin IgG antibody can be detected as early as day 4 of heparin treatment, which suggests preimmunization by antigens that mimic the PF4/heparin antibody complexes. It appears the preimmunization likely occurs as a physiological immune response to bacterial infections but in the presence of heparin is converted to a pathological response.¹⁸ A spontaneous HITTS syndrome has been described in individuals who have not received heparin.¹⁹ These individuals had anti-PF4/heparin IgG antibodies that caused in vitro platelet activation even in the absence of heparin.

Laboratory confirmation of HITTS may be difficult. Pretest probability assessment is important to avoid over diagnosis in patients with thrombocytopenia due to other causes. The most widely used assessment tool ("4Ts") uses easily available clinical information²⁰ (Table 26-5). If there is intermediate or high probability, initial immunoassays, such as ELISA, are used to screen for the presence of antibody in patient serum and to quantitate antibody amount by optical density (O.D.) measurement. The higher the O.D., the more likely HITTS is present. To ensure not missing cases, most laboratories use a cut-off nearer the upper limit of normal, 0.4 O.D. units, to ensure maximal sensitivity, but this decreases the specificity. An O.D. >1.0 is usually present in confirmed cases. Confirmatory testing with a functional assay demonstrating platelet

TABLE 26-5 Heparin-Induced Thrombocytopenia—4Ts Pretest Probability

4Ts 2 Points 1 Point 0 Points

Thrombocytopenia Platelet nadir >20 or fall Platelet nadir 10 to 19 or Platelet nadir of <10 or fall from baseline $>50\%$ fall to 30% to 50% from baseline to $<30\%$ from baseline

Timing of platelet Days 5 to 10 Consistent with 5 to 10 days but <4 days without recent decline missing counts or after day 10 heparin exposure

Thrombotic problems New thrombosis, Progression or recurrence of None skin necrosis, thrombosis, unproven thrombosis acute systemic reaction to heparin

Other causes of None obvious Possible Definite

Thrombocytopenia

Low score 0-3 points intermediate score 4-5 points High score 6-7 points
Reprinted from Lo GK, et al. Evaluation of pretest clinical score (4Ts) for the diagnosis of heparin-induced thrombocytopenia in 2 clinical settings. *Journal of Thrombosis and Hemostasis*. 2005;4:759.

activation in the presence of heparin such as ¹⁴C serotonin release; ATP release by lumiaggregometry; or other methods are then used if the initial immunological testing is positive.

In patients with suspected HITTS, heparin must be discontinued. The platelet count should return to normal within 4 to 6 days off heparin. Alternative anticoagulation is mandatory with or without initial thrombotic complications in any individual in whom the diagnosis of HITTS is strongly suspected due to the continued high risk of thrombosis off heparin. In patients who have life- or limb-threatening thrombosis, there are a limited number of choices for intravenous anticoagulation that all have potential hemorrhagic risk.

ADVANCED CONTENT

Argatroban is a synthetic direct thrombin inhibitor approved for treatment in this setting. Bivalirudin, an intravenous synthetic derivative of hirudin, a leech salivary gland protein, has been used off-label and has a somewhat shorter half-life than argatroban. Fondaparinux, which is the synthesized pentasaccharide portion of the heparin molecule, does not appear to cross-react with heparin in the manner that low molecular weight heparins can in promoting the clinical syndrome and is used off-label for this indication. Fondaparinux is administered subcutaneously, is less expensive, and does not require monitoring. The newer direct oral anticoagulants, such as dabigatran, a thrombin inhibitor, and the anti-Xa inhibitors (rivaroxaban and apixaban) are other anticoagulant alternatives. Bleeding may occur with all of these treatments but the parenteral direct thrombin inhibitors have short plasma half-lives, so their effects resolve relatively quickly, and there are reversal agents for the oral direct anticoagulants, idarucizumab (Praxbind) for dabigatran and andexanet alfa (Andexxa) for rivaroxaban and apixaban. Fibrinolytic therapy may play a treatment role to dissolve thrombus, especially in individuals who develop neurovascular compromise related to their thrombosis.

Acquired Secondary Immune-Mediated Thrombocytopenia

Lymphoproliferative Disorders/Collagen Vascular Disorders

Lymphoproliferative disorders such as Hodgkin's disease, chronic lymphocytic leukemia, and non-Hodgkin's lymphomas have been reported with an ITP-like thrombocytopenia associated with decreased platelet survival. In systemic lupus erythematosus (SLE), roughly 14% of the patients develop thrombocytopenia resembling ITP during the course of the disease. The hematologic manifestations of SLE, which include immune-mediated thrombocytopenia and thrombocytopenia secondary to bone marrow suppression, may precede the other clinical manifestations of the disease. Thrombocytopenia in SLE responds well to corticosteroid therapy.

Infections Acute viral infections may transiently impair megakaryopoiesis without a reduction in marrow cellularity.

Chronic viral infections such as human immunodeficiency virus (HIV) or hepatitis may lead to marrow hypocellularity and, subsequently, to thrombocytopenia in affected individuals. Thrombocytopenia as a result of acute or chronic viral, bacterial, or parasitic infections may occur due to an immunological mechanism. Viral infections such as mononucleosis, mumps, and rubeola can cause very severe thrombocytopenia. In bacterial sepsis, thrombocytopenia may be present with or without disseminated intravascular coagulation (DIC). Malaria is frequently associated with thrombocytopenia as a result of increased platelet destruction and splenic sequestration.

An array of hemostatic complications has been described in association with HIV infection. The most common hemostatic abnormality in these patients is thrombocytopenia. The incidence of thrombocytopenia appears to vary according to the stage of the disease. There appears to be a correlation between CD4+ T cell depletion, viral load in plasma, and the occurrence of thrombocytopenia. Many affected patients also have active hepatitis infection. Most patients with HIV-related thrombocytopenia do not have significant bleeding. Hemorrhagic complications may occur but are difficult to predict based solely on the platelet count.

The pathogenesis of HIV-related thrombocytopenia appears to be heterogeneous.²¹ An immune etiology is suggested by studies that have shown diminished *in vivo* platelet survival. Detection of platelet-associated IgG and circulating immune complexes that contain antiplatelet antibodies provides support for immune destruction in HIV-related thrombocytopenia. The pattern of IgG subclasses found in HIV-infected patients, as well as the level of immune complexes on platelet surfaces in HIV disease, are significantly different from patients with "classic ITP" (see Table 26-4). Immunohistochemical markers show increased CD8+ T cells from the spleens of patients with HIV-related immune thrombocytopenic purpura. However, the finding that thrombocytopenia does not frequently occur in infants of HIV-infected mothers with thrombocytopenia suggests an immune etiology may not be the principal cause. Viral infection of hematopoietic cells, altered marrow microenvironment, or dysfunction of the reticuloendothelial system contribute to ineffective thrombopoiesis in HIV-related thrombocytopenia. Development of marrow fibrosis or marrow involvement by AIDS-related lymphoma may also lead to thrombocytopenia. These factors may be more important in producing HIV-related thrombocytopenia.

Treatment with antiretroviral therapy alone is often effective. HIV-related thrombocytopenia, when more severe or associated with bleeding, is treated similarly to ITP. Intravenous IgG and anti-D globulin have been used successfully. Corticosteroids may be effective but have the potential to increase the risk of infection in these immunosuppressed individuals. Thrombopoietin receptor agonists are often preferred over other immunosuppressive treatment modalities such as rituximab or splenectomy, which also increase risks for infection.

Vaccine-Related Immune Thrombocytopenia As viral infections may cause thrombocytopenia, vaccinations against

viral illness may also cause thrombocytopenia. Most vaccinations against viruses rarely cause immune thrombocytopenia. Measles vaccination-related ITP is estimated to occur at a rate of 1 to 3 cases per 100,000 doses. Recently, COVID-19 vaccinations have been reported to cause thrombocytopenia by two different mechanisms. The mRNA vaccines by Pfizer and Moderna are reported to cause a rapid onset, severe thrombocytopenia, which responds to treatment for ITP.²² The adenovirus vector vaccines, by Johnson & Johnson and by AstraZeneca, may cause thrombocytopenia with thrombosis that is unusual, such as cerebral or splanchnic veins. Antibodies directed against platelet factor 4 activate platelets in a similar fashion as HITTS but in the absence of prior heparin exposure.²³ Both types of vaccine-related immune thrombocytopenia are rare, estimated at about 0.80 cases per million doses of the mRNA vaccines and 3.6 cases per million doses of the adenovirus vector vaccines.

Thrombotic Thrombocytopenic Purpura Thrombotic thrombocytopenic purpura (TTP) is a rare and sometimes fatal syndrome associated with thrombocytopenia and microangiopathic hemolytic anemia^{24,25} (Fig. 26-5). Despite being recognized since 1924, the pathogenesis of this syndrome remains poorly understood. Both inherited and sporadic acquired nonfamilial forms of the syndrome occur. This disorder was first described as a pentad of signs and symptoms that included thrombocytopenia, microangiopathic hemolytic anemia, fever, neurological abnormalities, and renal dysfunction. As TTP is recognized more readily, it has become clear that thrombocytopenia and microangiopathic hemolytic anemia characterize this disorder, and the fluctuating neurological abnormalities, fever, and renal dysfunction occur less frequently. The diagnosis should be suspected in any individual who presents acutely with thrombocytopenia, and consideration should be given to initiation of appropriate treatment even in the absence of other signs or symptoms while awaiting laboratory confirmation of the diagnosis. Hyaline microthrombi are the characteristic pathological feature and are found in multiple organs on biopsies or at autopsy. However, microthrombi are not solely diagnostic for TTP, as they occur in other microangiopathic processes such as DIC (Fig. 26-6).

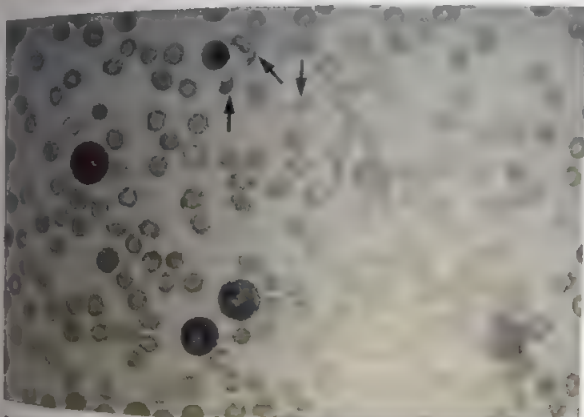


FIGURE 26-5 Microangiopathic hemolytic anemia. Note the presence of schistocytes (arrows) and nucleated red blood cell (top border).

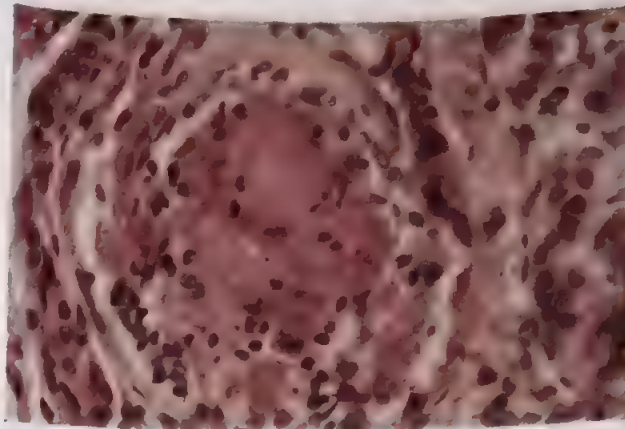


FIGURE 26-6 Renal biopsy from a patient with thrombotic thrombocytopenic purpura (TTP) showing glomerular deposits of platelet-fibrin microvascular occlusion.

The coagulation screening tests and D-dimer assay are normal in TTP, in contrast to DIC, where they are abnormal (see Chapter 28).

The exact pathogenic mechanisms responsible for this disorder remain uncertain. Endothelial cell damage, possibly secondary to drugs or other agents, may be critical to the development of vascular injury. A number of drugs have been reported to induce thrombotic microangiopathies resembling TTP.²⁶ Once endothelial cell damage occurs, inhibition of fibrinolysis and deficiency of prostacyclin (PGI_2) synthesis may then lead to platelet aggregation and thrombosis. The large von Willebrand factor multimers that are secreted by damaged endothelial cells may then persist in circulation and promote development of microvascular thrombosis. The presence of platelet microparticles due to platelet destruction may increase the thrombotic tendency. A genetic predisposition or an underlying disorder may be necessary for expression of the disease, but this has not been proven except for the familial variant of the disorder.

In the rare inherited familial variant of TTP, there is now evidence that the protease in vascular endothelial cells, which is responsible for cleaving von Willebrand multimers (ADAMTS 13), is defective or absent.²⁷ This disorder is caused by ADAMTS 13 mutations with deficient production of the protease. This form of TTP usually appears in infancy or early childhood and is treated with periodic plasma infusions to maintain remissions by replacing the ADAMTS 13 deficiency in plasma. A recombinant form of ADAMTS 13 (SHP 655) is currently in clinical trials for treatment of familial TTP.

In the more common nonfamilial acquired cases, autoantibody against the ADAMTS 13 protease is present that inhibits the protease,²⁸ either by neutralizing the proteolytic activity or enhancing the clearance of the protease. Women appear to be affected more often than men, with a female-to-male ratio of 3:2. The peak incidence occurs between the third and fourth decades of life. Patients who are pregnant, have viral infections, ingest certain drugs such as clopidogrel, or have autoimmune disorders appear to be predisposed

to development of TTP. TTP may also occur as a complication of antineoplastic chemotherapy or allogeneic stem cell transplantation, and in those cases, ADAMTS 13 levels are not significantly decreased and prognosis tends to be poor with standard treatments.

Common Clinical Findings

Patients with TTP initially present with nonspecific symptoms of malaise, weakness, fatigue, fever, or abdominal pain. The clinical spectrum of TTP can vary and simulate other thrombotic microangiopathies. Neurological dysfunction and renal abnormalities are part of the classic pentad but need not be present to make the diagnosis. The degree of severity of these signs and symptoms can be quite variable; fever is typically less than 38.3°C, and the renal abnormalities may be as mild as proteinuria or microscopic hematuria. Overt renal failure requiring dialysis is uncommon in TTP, in contrast to hemolytic uremic syndrome (HUS). Neurological manifestations can be as mild as headaches or as severe as coma, seizures, or obtundation. Abdominal pain, pancreatitis, and gastrointestinal bleeding may also be associated findings. Any organ system may become involved in TTP; however, symptomatic pulmonary and cardiac involvement are unusual. Pericarditis is rare in TTP, but diffuse cardiac ischemia mimicking pericarditis with diffuse ST segment elevation on electrocardiogram (ECG) can be seen and is typically fatal despite appropriate treatment.

Laboratory Testing and Results

Laboratory features of TTP are those of a severe microangiopathic hemolytic anemia with schistocytes, reticulocytosis, and nucleated red blood cells on the peripheral blood smear. Signs of hemolysis are reflected by increases in LDH and indirect bilirubin, decreased haptoglobin levels, hemoglobinemia, and hemoglobinuria. There is severe thrombocytopenia and evidence of decreased peripheral platelet survival despite megakaryocytic hyperplasia in the bone marrow. However, early in the course of TTP, schistocytes and nucleated red blood cells may not be prominent, and severe thrombocytopenia may be the predominant finding. The prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen level, and D-dimer are generally normal in patients with TTP. Slight elevations in the fibrinogen degradation products (FDPs) have been reported. ADAMTS 13 levels are severely depressed (less than 10%) in most individuals with TTP and may predict a better potential for response than for individuals with normal ADAMTS 13 levels. Presence of an autoantibody to the ADAMTS 13 protease is consistent with an acquired TTP. The clinical course of a patient does not always parallel the change in the ADAMTS 13 levels. Individuals may rapidly normalize ADAMTS 13 levels in plasma but remain clinically affected by TTP while others may have a remission and still have low plasma ADAMTS 13 levels. Once an individual is in remission, monitoring the ADAMTS 13 activity levels appears useful in predicting relapse.

Treatment

Before the development of newer treatment modalities such as plasma exchange, the mortality in TTP exceeded 90%.²⁴ Plasma exchange is presently the standard of care for

treatment of acute TTP. With the use of plasma exchange there has been a significant improvement in the survival of patients with TTP. Survival rates of 80% to 85% are reported, but relapses occur in about 40% of patients, with fatal outcomes in patients with refractory disease.²⁵ The frequency of plasma utilized, and type of plasma for exchange have not been standardized, although most experts support daily exchanges of at least one complete plasma volume utilizing fresh frozen plasma. The length of time plasma exchange should be continued is also not clearly defined, although most clinicians support continuation of the plasma exchange until the platelet count and LDH level have both normalized. As this is now known to be an autoimmune disorder, use of immunosuppressive therapy is usually considered. Corticosteroid therapy when used as the sole therapeutic agent is usually ineffective, except perhaps in very early relapses. Theoretically, corticosteroids in addition to plasma exchange may offer additional benefit, although the amount of corticosteroids needed and the length of therapy are not clearly defined. Cyclosporin A has also been used; however, a report of the use of prednisone compared with cyclosporin A demonstrated that prednisone suppressed anti-ADAMTS 13 antibodies and improved ADAMTS 13 activity better in the first month after stopping plasma exchange, but did not appear to decrease subsequent relapse rates. Caplacizumab is a monoclonal antibody that targets the A1 domain of von Willebrand factor that interacts with platelet GP IIb to block the platelet vWF interaction and prevent formation of microthrombi. Caplacizumab appears to reduce recurrences within 30 days of stopping plasma exchange but not decrease late recurrences. There have been numerous case reports of the effectiveness of rituximab treatment in refractory TTP and one clinical trial of the effectiveness in preventing relapses. Currently, many clinicians are now using rituximab after clinical remission has occurred after the cessation of plasma exchange to preemptively prevent relapses, after an initial episode, and for patients in clinical remission whose ADAMTS 13 activity level decreases before other signs of relapse. Patients should be monitored closely after discontinuation of plasma exchange for evidence of relapse, which is common. Platelet transfusions should be avoided unless the patient is overtly bleeding, because platelet transfusions may accelerate the microangiopathy and have been temporally related to deterioration and death.

In patients who do not respond to plasma exchange or who relapse on plasma exchange, there is no standard treatment intervention, but several options can be considered. The literature has case reports of many treatments that are effective in small numbers of patients. Rituximab may be considered if not previously shown to be ineffective. Splenectomy has been considered as a therapeutic intervention once patients fail plasma exchange or for patients prone to relapses of the disorder. The majority of patients undergoing splenectomy have also received corticosteroids and blood products, which may also increase the response rate. Immunosuppressive chemotherapy drugs, such as vincristine or cyclophosphamide, have been reported to induce remission. Antiplatelet drugs such as aspirin, dipyridamole, and sulfipyrazone have been used in

the past (often these antiplatelet drugs are used in variable combinations, making their efficacy difficult to evaluate). Because patients initially present with thrombocytopenia, antiplatelet agents may increase the risk of hemorrhage. Anticoagulant therapy, including heparin and dextran, has been used in the past but has shown no benefit.

Hemolytic Uremic Syndrome HUS resembles TTP pathologically, but unlike TTP, which usually affects adults, typical HUS is more commonly seen in children, presenting with diarrhea, usually due to *Escherichia coli* or *Shigella* bacterial infections that secrete toxins causing endothelial cell damage. Typical childhood HUS usually takes a milder course than TTP with a mortality between 1% and 5%.²⁹ The prognosis of adult-onset typical HUS is worse than that of childhood-onset HUS, with not a higher mortality rate and higher incidence of long-term morbidity from permanent renal damage.³⁰ Table 26-6 compares the characteristics of TTP and typical HUS. The pathological thrombi in HUS are almost always limited to the glomerular capillaries and afferent arterioles of the kidney.

Common Clinical Findings

Fever, hypertension, and renal failure are common findings, whereas neurological manifestations are usually less common and less severe. Severe neurological problems or evidence of systemic thrombi are more likely to suggest the diagnosis of TTP. Secondary HUS may be due to other bacterial or viral infections, organ transplantation, autoimmune disease, pregnancy, or cytotoxic chemotherapy. These conditions can cause direct cell damage and enhance complement activation.³¹ A hereditary form of HUS, "atypical HUS," is due to congenital deficiencies of complement regulators and complement proteins. This disorder is more commonly diagnosed in adults and has an unfavorable prognosis but can present in childhood. Presenting clinical symptoms and signs are similar for all three of these types of HUS, and some patients that appear to have typical or secondary HUS may also have mutations or autoantibodies related to complement regulation. A recent updated classification of thrombotic microangiopathies and treatment of complement gene variant-mediated microangiopathy has been proposed.³²

Treatment

Therapeutic management of typical HUS is usually conservative. Supportive therapy may include fluid replacement,

hemodialysis, antihypertensive therapy, and red blood cell and platelet transfusions. Unlike TTP, plasma exchange is usually not necessary in typical Shiga toxin-mediated HUS. However, in secondary HUS and atypical HUS, plasma exchange therapy is indicated as initial therapy, except in patients with HUS secondary to pneumococcal infection as this treatment may exacerbate the disease. Atypical complement-mediated HUS may be treated with eculizumab, a monoclonal antibody to complement component C5, to reduce hemolysis and prevent chronic renal failure. A long-acting C5 inhibitor, ravulizumab, has recently become available for maintenance therapy. Due to a significant risk of meningococcal disease with complement inhibitor therapy, meningococcal vaccination is recommended before beginning this therapy.

Nonimmune Thrombocytopenia

Disseminated Intravascular Coagulation

Acute DIC is caused by many illnesses, sepsis, obstetric emergencies, and severe trauma, and may cause bleeding. Chronic DIC may be seen with cancer and may result in thrombosis rather than bleeding. Thrombocytopenia is usually seen in acute DIC; however, the platelet count may be normal or elevated in chronic DIC. In all instances, there appears to be accelerated platelet destruction in combination with coagulation factor consumption. DIC must be differentiated from other causes of microangiopathy causing thrombocytopenia because treatments differ. Pathogenesis and treatment of DIC is discussed in Chapter 28.

Pregnancy-Associated Thrombocytopenia

Thrombocytopenia may occur in pregnancy owing to coincidental development of disorders discussed earlier in this chapter, or it may occur as a consequence of the pregnancy.³³ Disorders resulting from pregnancy include gestational thrombocytopenia and thrombotic microangiopathies such as preeclampsia-eclampsia and HELLP syndrome (hemolysis, elevated liver enzymes, low platelet count).

Gestational Thrombocytopenia Mild thrombocytopenia with platelet counts of 50 to 80 × 10⁹/L may occur in about 8% of normal pregnant women. This most commonly develops in the third trimester of pregnancy and does not cause bleeding in the mother or infant. No treatment is necessary for this disorder. The platelet count returns to normal after delivery. Gestational thrombocytopenia shares features with TTP, but the finding that infants born to mothers with gestational thrombocytopenia do not have neonatal thrombocytopenia indicates this may not be immune-mediated. Gestational thrombocytopenia must be differentiated from other thrombocytopenic disorders of pregnancy such as eclampsia, HELLP syndrome, TTP, or TTP, because these disorders may cause harm to the mother or the fetus and require treatment to prevent maternal or fetal morbidity and mortality.

Preeclampsia-Eclampsia and HELLP Syndrome Preeclampsia is a relatively common disorder of pregnancy that causes hypertension, elevation of uric acid, and thrombocytopenia. Eclampsia is a more severe form of the disorder and may result in seizures and renal dysfunction; it also has a greater association with thrombocytopenia. A variant of

TABLE 26-6 Comparison of TTP and HUS

Characteristics	TTP	
Age at onset	Adult	Children
Organ involvement	Multiple	Kidney
Neurologic abnormalities		
Microangiopathic HA	Yes	Yes
Thrombocytopenia	Yes	Yes
Neurological abnormalities	Yes	Rare
Renal complications	Renal abnormalities	Renal failure

eclampsia termed **HELLP** syndrome (Hemolysis, Elevated Liver Enzymes, Low Platelets) is also described.³⁴ These disorders are thrombotic microangiopathies with clinical manifestations resulting from platelet activation and consumption from endothelial activation and cell injury. The more severe forms of these syndromes must be differentiated from TTP or HUS. Preeclampsia and eclampsia typically occur with the first pregnancy and usually occur in the last trimester, whereas TTP and HUS may occur at any time in pregnancy. Delivery of the fetus usually is effective in reversing the eclamptic disorders. Aspirin appears to be useful in treating preeclampsia. Corticosteroid treatment may be considered to improve fetal lung maturity if HELLP occurs early in the last trimester of pregnancy. However, when these disorders are severe or serious complications have developed, plasma exchange therapy is used because it may be difficult to differentiate eclampsia and HELLP from TTP or HUS.

Quantitative Platelet Disorders: Thrombocytosis

Thrombocytosis, defined as platelet count above the normal range, may be either a reactive process or due to a primary clonal **myeloproliferative neoplasm (MPN)**.³⁵ Reactive thrombocytosis, unlike thrombocytosis from a primary MPN, is rarely associated with bleeding and thrombotic complications, and the platelet count usually does not exceed $1000 \times 10^9/L$. The morphology of platelets in a reactive process is usually normal in contrast to those in an MPN, where the platelets are often large and dysplastic. Tests of platelet function are also typically normal in reactive thrombocytosis. Bone marrow examination may demonstrate increased numbers of megakaryocytes; however, in contrast to MPNs, the megakaryocytes are neither clustered nor dysplastic with reactive thrombocytosis.

CRITICAL THINKING QUESTION

26-2 If thrombocytosis is an excess of platelets, what is the expected clinical symptom in patients with this disorder?

Primary Thrombocytosis

Because all of the MPNs are characterized by a clonal autonomous proliferation of a pluripotent stem cell, they can all be associated with thrombocytosis. This proliferation of platelets is independent of the influence of TPO. Bleeding, thrombosis, and platelet function defects can also be seen with any of the MPNs. (The MPNs as they relate to specific platelet defects are discussed later in the section on acquired qualitative platelet disorders.) Patients with essential thrombocythemia typically have the highest platelet counts of all of the MPNs, often exceeding $1000 \times 10^9/L$. Primary myelofibrosis, polycythemia rubra vera, and chronic myelogenous leukemia (CML) are associated with milder degrees of thrombocytosis. Based on observations of patients with reactive thrombocytosis and normal platelet function, bleeding and thrombotic complications are uncommon. However, thrombocytosis alone is not

the sole factor in the development of bleeding and thrombosis in the MPNs. These complications are suggested to result from qualitative platelet defects. Lowering of extreme thrombocytosis with hydroxyurea or thrombocytapheresis may improve the bleeding tendency in MPNs.

Reactive Thrombocytosis

Reactive thrombocytosis may be attributed to many causes and may be either a transient or chronic process. Inflammatory cytokines such as interleukin-6 and -11 are thought to be responsible for producing this response. Thrombopoietin levels in plasma are reduced due to high platelet counts.

Thrombocytosis is a common finding associated with recovery from acute hemorrhage. The platelet count is generally elevated within a day or so after hemorrhage as a result of increased marrow stimulation. Similar responses may be seen after therapeutic phlebotomy for treatment of polycythemia vera (PV) or hemochromatosis (see Chapters 7 and 19).

Iron-deficiency anemia has classically been associated with mild thrombocytosis that does not usually exceed $750 \times 10^9/L$. This associated thrombocytosis may help to differentiate iron deficiency from other causes of red blood cell microcytosis that are not typically associated with thrombocytosis. Repletion of iron stores corrects the platelet count to normal.

Thrombocytosis is also associated with underlying malignancy and with chronic inflammatory or infectious processes.

Thrombocytosis can be seen postoperatively after almost any surgical procedure but is most common and pronounced after splenectomy. Within the first 2 weeks, platelet counts may increase to as much as two to six times preoperative levels and then decline to a higher normal or slightly above normal level over a period of months after splenectomy. Platelet survival has been documented to be normal. It has been suggested that the thrombocytosis seen after splenectomy is caused by increased platelet production, because elimination of the splenic pool can account for no more than a 50% rise in the platelet count.

ADVANCED CONTENT

Certain drugs may also induce thrombocytosis or assist in platelet recovery after an episode of thrombocytopenia. Administration of epinephrine will cause a rapid but transient increase in platelet count secondary to platelet release from the spleen. Recovery of marrow suppression from alcohol or chemotherapeutic agents will often result in thrombocytosis. Recombinant interleukin-11 (oprelvekin) has been used to aid in platelet recovery after chemotherapy administration to prevent bleeding but has not been very effective and has not helped in other thrombocytopenic states, so it is now rarely used clinically. Recombinant TPO was studied in normal individuals but resulted in anti-TPO antibodies and refractory thrombocytopenia. However, this led to the development of TPO mimetics, romiplostim, eltrombopag, and avatrombopag. These agents are now used clinically for treatment of refractory ITP.

Qualitative Platelet Disorders

Congenital Disorders of Platelet Function

Congenital disorders of platelet function are rare diseases caused by various genetic mutations and can be broadly classified based on the platelet function or response that is abnormal (Box 26-3). This classification currently includes:

1. Platelet surface membrane defects
2. Platelet release or secretion defects
3. Platelet coagulant activity defects
4. Defects associated with abnormal platelet-coagulant protein interactions

Numerous syndromes have been described within these categories.^{36,37} Some of these disorders also exhibit thrombocytopenia in addition to platelet dysfunction. Cutaneous, skeletal, and other congenital abnormalities may also be present in these disorders. The more common congenital disorders as they relate to abnormal platelet function are listed in Tables 26-3 and Box 26-3. As noted in Chapter 25, the platelet surface includes a glycocalyx containing various glycoproteins that function as receptors to bind molecules or proteins and transmit signals to the interior of the platelet to effect platelet reactions. Glanzmann's thrombasthenia and Bernard-Soulier syndrome are congenital disorders with surface glycoprotein deficiencies that result in platelet dysfunction. The laboratory abnormalities in these syndromes are noted in Table 26-7.

As described in Chapter 25, platelet glycoprotein receptors transmit signals intracellularly to cause release of platelet granule substances and platelet shape change. Phospholipases (A and C) are liberated from the internal surface of the platelet membrane in response to platelet agonists, release cyclo-oxygenase, and cause an increase in intracellular calcium levels. This produces centralization of granules and fusion of granule membrane with the open canalicular system to transport granule proteins to the environment to allow adhesion and aggregation. The disorders of platelet release primarily involve absence of platelet granules or defective enzymatic pathways. Storage pool deficiencies have absence of alpha or dense granules, whereas the release disorders have normal amounts of platelet granule

BOX 26-3 Congenital Disorders of Platelet Function

Platelet Surface Membrane Defects

- Glanzmann's thrombasthenia
- Bernard-Soulier syndrome

Platelet Release or Secretion Defects

- Storage pool deficiency (granule defects)
- Primary secretion defects (enzymatic pathway defects)

Platelet Coagulant Activity Defects

- Defects associated with abnormal platelet coagulant protein interaction
- von Willebrand Disease

TABLE 26-7 Comparison of Glanzmann's Thrombasthenia and Bernard-Soulier Syndrome

Laboratory Test	Glanzmann's Thrombasthenia	Bernard-Soulier Syndrome
Platelet count	Normal	Decreased
Platelet morphology	Normal	Giant platelets
Platelet aggregation		
ADP	Abnormal	Normal
Thrombin	Abnormal	Abnormal
Collagen	Abnormal	Normal
Epinephrine	Abnormal	Normal
Ristocetin	Normal	Abnormal
Clot retraction	Abnormal	Normal
Platelet glycoprotein defect	GPIIb/IIIa	GPIb/IX/V

contents. The bleeding tendency and laboratory findings in these disorders are similar.

CRITICAL THINKING QUESTION

26-3 Is it feasible that patients with a qualitative platelet disorder could have a normal platelet count?

Platelet disorders with failure to generate thrombin are considered to have defects in platelet anticoagulant activity. Very few patients with this problem have been described. In one individual extensively studied, there was a failure of platelet microvesiculation in response to stimulation, leading to decreased Va-Xa and VIIa-IXa binding that slowed the normal coagulant response. Individuals with these disorders do not demonstrate abnormal platelet adhesion or aggregation.

Von Willebrand disease is caused by a plasma protein deficiency or protein dysfunction with abnormal platelet-coagulant protein interactions that mimics primary platelet disorders by its pattern of mucosal bleeding. (See Chapter 27.)

Platelet Surface Membrane Defects

Glanzmann's Thrombasthenia Glanzmann's thrombasthenia is a rare autosomal-recessive disorder of platelet function caused by an absence or deficiency of the membrane GPIIb/IIIa complex. GPIIb/IIIa mediates the binding of fibrinogen, von Willebrand factor (vWF), and fibronectin to activated platelets. GPIIb/IIIa also serves to connect adhesive proteins to contractile proteins of the platelet after activation, thereby facilitating clot retraction. These two proteins exist within the platelet membrane as heterodimers. Calcium is required for stabilization of the complex. GPIIb/IIIa must exist as a complex to function as a ligand to bind fibrinogen. Both megakaryocytes and platelets express the GPIIb/GPIIIa complex.

The gene that codes for GPIIb and GPIIIa is located on chromosome 17. Gene deletion and gene rearrangement

of GPIIb may account for the phenotypic defects seen in Glanzmann's thrombasthenia.

Common Clinical Findings

Clinical manifestations of Glanzmann's thrombasthenia are quite variable, ranging from minor bruising to severe and potentially fatal hemorrhages, with the severity of bleeding consistent within single families. Although uncommon, Glanzmann's thrombasthenia occurs with greater frequency than Bernard-Soulier syndrome, another surface glycoprotein abnormality. Glanzmann's thrombasthenia appears to cluster in ethnic populations where consanguinity is prevalent. Bleeding is most commonly from mucosal surfaces and includes easy bruisability, epistaxis, spontaneous gingival bleeding, prolonged bleeding from minor cuts, and menorrhagia. Gastrointestinal hemorrhages are less common. Facial petechiae and subconjunctival hemorrhages may be seen in infants associated with crying. The formation of deep hematomas and recurrent hemarthroses that are typical features in hemophilia are not present in Glanzmann's thrombasthenia.

Diagnostic Criteria

Criteria for diagnosing Glanzmann's thrombasthenia include:

1. An autosomal-recessive trait with clinical manifestations expressed in homozygotes only
2. Normal platelet count and normal platelet morphology
3. Abnormal response to collagen-ADP with PFA-100 testing
4. Absent platelet aggregation to ADP, thrombin, collagen, and epinephrine, with normal platelet agglutination to ristocetin (Fig. 26-7)
5. Flow cytometric assessment of GPIIb/IIIa surface protein, revealing deficiency or absence
6. Abnormal light transmission aggregometry
7. Abnormal clot retraction

Treatment

Prevention of bleeding with good dental care and avoidance of antiplatelet drugs is important. Patients with Glanzmann's thrombasthenia who present with severe bleeding episodes require platelet transfusions to replace dysfunctional platelets. Supportive therapy should be used judiciously, because patients may develop alloantibodies to GPIIb and GPIIb on the transfused platelets or anti-HLA antibodies and become refractory to platelet transfusions. Antifibrinolytic agents such as EACA or tranexamic acid may help in controlling hemorrhage, especially from the nose and mouth where fibrinolytic activity is prominent. Topical measures such as thrombin or pressure packing may be effective. DDAVP has been used but has not been helpful. Estrogen therapy in the form of birth control pills is especially useful in controlling menorrhagia. Recombinant factor VIIa has been successfully used, but thromboembolism has occurred in one reported case after discontinuation of the recombinant factor VIIa treatment.

Bernard-Soulier Syndrome Bernard-Soulier syndrome is a rare autosomal-recessive bleeding disorder caused by a deficiency of the platelet GPIb/IX complex. GPV, which is associated with the GPIb/IX complex on the platelet surface and is a thrombin substrate, has also been documented to be deficient in Bernard-Soulier platelets. Gene mutations of GPIb and GPIX, but not GPV, appear to be the cause of this disorder. An acquired form of the disorder has been reported due to autoantibodies to these platelet glycoproteins. As discussed in Chapter 25, GPIb/IX/V plays a major role in various hemostatic events. GPIb/IX/V is the platelet receptor involved in the vWF-dependent contact adhesion of unactivated platelets to exposed subendothelium at high shear rates and for the binding of platelets to fibrin. It also serves as a high-affinity thrombin-binding site and regulates platelet shape

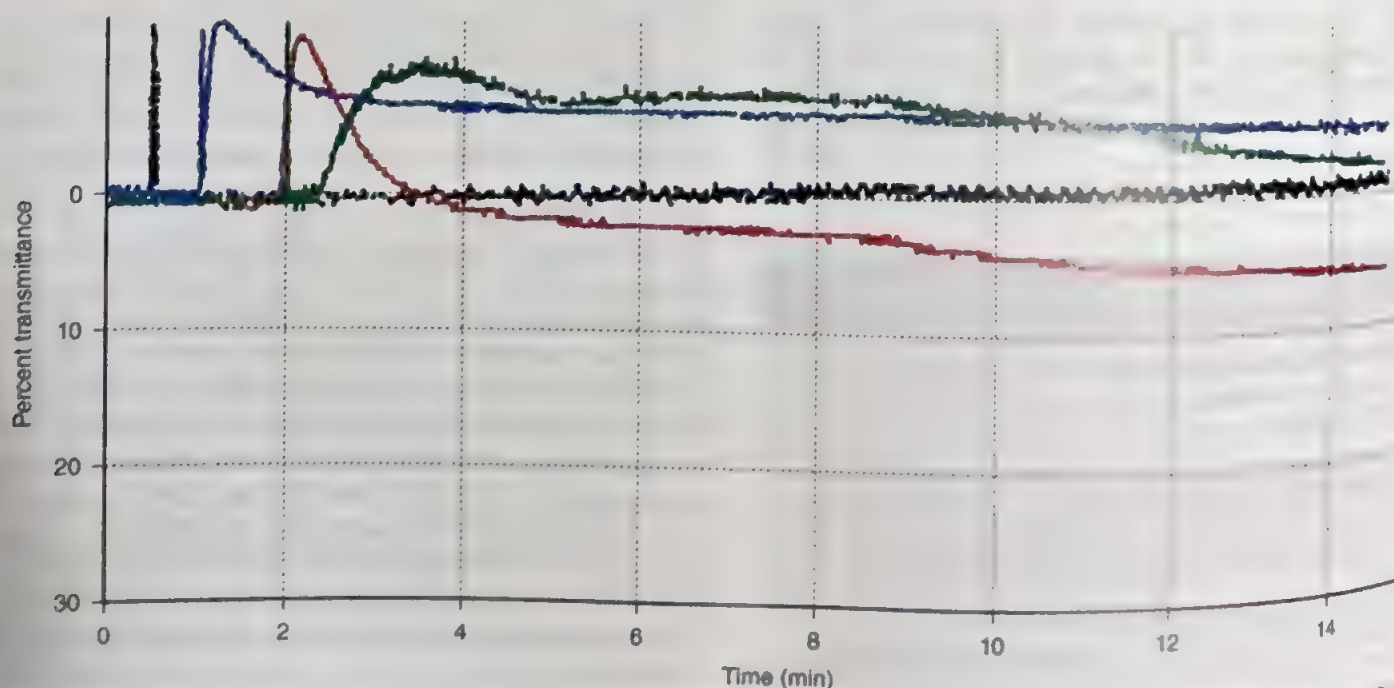


FIGURE 26-7 This graph depicts the lack of platelet aggregation to epinephrine, 10 µM (blue); ADP, 5 µM (black); collagen, 2 mcg/mL (red); and arachidonic acid, 0.5 mcg/mL (green)—typical of a patient with Glanzmann's thrombasthenia.

and reactivity. Because the platelet membrane is attached to the cytoskeleton via GPIb, the loss of normal membrane-cytoskeletal function may account for the abnormal platelet morphology seen in Bernard-Soulier platelets. Bleeding in Bernard-Soulier syndrome is due to a combination of hemostatic defects including thrombocytopenia, abnormality of platelet-von Willebrand factor interaction important for platelet adhesion, abnormality of platelet-thrombin interaction, and abnormality of platelet coagulant activity.

Common Clinical Findings

Patients with Bernard-Soulier syndrome present with the typical symptoms of a primary hemostatic disorder with varying severity. Gingival bleeding, epistaxis, purpura, menorrhagia, and gastrointestinal bleeding are the typical hemorrhagic manifestations. Symptoms occur early in life and tend to decrease with age.

Laboratory Testing and Results

Laboratory abnormalities in patients with Bernard-Soulier syndrome include normal or moderately reduced platelet counts with large irregularly shaped platelets noted on the peripheral smear. The platelets seen on peripheral blood smear may be large enough to resemble lymphocytes. Normal numbers of megakaryocytes are found on bone marrow examination. Platelet aggregation is normal with ADP, epinephrine, and collagen, and is reduced with thrombin. Ristocetin-induced platelet aggregation is absent in Bernard-Soulier syndrome, similar to von Willebrand disease. However, the deficient ristocetin-induced aggregation defect is corrected by the addition of normal plasma in von Willebrand disease, but not in Bernard-Soulier syndrome, where the deficiency of GPIb/IX/V (which is the receptor for vWF) is the cause of the aggregation defect. The differences in platelet aggregation tests with various platelet agonists among von Willebrand disease, Bernard-Soulier syndrome, and Glanzman's thrombasthenia are depicted in Table 26-8. Crossed immunoelectrophoresis or flow cytometry of platelet membrane glycoproteins should demonstrate a decrease in GPIb, GPIX, and GPV to confirm a diagnosis of Bernard-Soulier syndrome.

Heterogeneity among the glycoprotein abnormalities in Bernard-Soulier indicates that there are multiple genetic defects. This has been confirmed with the discovery of at least 112 different mutations in GPIb α , GPIb β , and GPIX subunits.²⁸ Heterozygotes present with recognizable abnormalities such as occasional large platelets on the peripheral smear, with a history of bleeding yet few serious clinical problems. Homozygotes present with abnormal platelet function and morphology, thrombocytopenia, and hemorrhagic disease.

Diagnostic Criteria

Specific criteria for the diagnosis of this bleeding syndrome have been established. These criteria are:

1. An autosomal trait with clinical manifestations expressed in homozygotes (or double heterozygotes with combined genetic abnormalities of GPIb, GPV, and GPIX)
2. Normal platelet count or moderate thrombocytopenia (despite a normal number of marrow megakaryocytes)
3. A significant number of giant or large platelets present on the peripheral blood smear
4. Absent platelet agglutination in response to human vWF and ristocetin
5. Normal platelet aggregation in response to ADP, collagen, and epinephrine, with reduced aggregation in response to thrombin
6. Normal factor VIII coagulant activity (FVIII:C) and vWF antigen

Treatment

Affected individuals who present with active bleeding should be treated with red blood cell transfusions to replace blood loss and an antifibrinolytic agent such as EACA or tranexamic acid. Antifibrinolytic agents do not correct the defect but allow the primary hemostatic plug to remain intact at the site of injury. DDAVP and estrogen therapy may be helpful in controlling bleeding. In instances in which bleeding is not controlled by these measures, platelet transfusions may be necessary; however, these should be used judiciously to avoid alloimmunization to GPIb. There has been successful use of recombinant factor VIIa to treat bleeding in this disorder.

Platelet Release (Secretion) Defects

Storage Pool Deficiencies (Granule Defects) Storage pool deficiencies are classified according to an analysis of the granule proteins and the morphological appearance of the platelets. Most commonly, a decrease in platelet dense granules is present, with decreased amounts of secretable ADP, adenosine triphosphate (ATP), calcium, and serotonin (delta [δ] storage pool deficiency). In other patients, alpha (α) and dense granules are decreased with decreases in amounts of α granule proteins such as platelet factor 4, beta (β)-thromboglobulin, and platelet-derived growth factor as well as the dense granule proteins are noted ($\alpha\delta$ storage pool deficiency). Other patients have a decrease in α granules only, with normal dense granules and normal amounts of dense granule constituents (α storage pool deficiency). Deficiency of the α granules leads to an agranular appearance of platelets on the Wright-stained peripheral blood smear and has been termed *gray platelet syndrome*. The

TABLE 26-8 Comparison of Platelet

	ADP	Collagen	Thrombin	Epinephrine	Ristocetin	vWF
vWD	+	+	+	+	+	+
BSS	+	+	+	+	-	-
GT	-	-	-	-	-	N/A

vWD = von Willebrand disease; BSS = Bernard-Soulier syndrome; GT = Glanzmann's thrombasthenia

Quebec platelet disorder is reported to have a deficiency of α granule proteins related to excessive proteolysis. Platelet factor 3 (PF3) activity is reduced in these disorders and appears related to the defects in platelet aggregation.

The inheritance pattern of these disorders has not been well defined but in some instances appears to be an autosomal dominant pattern. Patients with storage pool deficiency have a mild to moderate mucosal bleeding tendency. Easy bruising, epistaxis, menorrhagia, postpartum bleeding, and bleeding after dental extractions or tonsillectomy are often present. Gastrointestinal bleeding is uncommon and hemarthrosis is not present. Storage pool deficiencies have been found in association with other congenital abnormalities (see Table 26-3). The Hermansky-Pudlak syndrome describes patients with oculocutaneous albinism and dense granule storage pool deficiency. Individuals with Chédiak-Higashi syndrome also have oculocutaneous abnormalities, including ocular albinism and characteristic silver-gray hair with dense granule storage pool deficiency. Other congenital abnormalities, including Wiskott-Aldrich syndrome and the syndrome of thrombocytopenia with absent radii (TAR syndrome), have been described to have an associated platelet storage pool deficiency.

Laboratory Features of Storage Pool Deficiencies

Often, these disorders have normal laboratory results with PFA-100, plasma coagulation and factor assays. Platelet aggregation testing in δ storage pool deficiency demonstrates normal primary wave aggregation but absent or reduced secondary wave aggregation in response to ADP and epinephrine. Collagen-induced aggregation is reduced at lower but not high collagen concentrations. High concentrations of thrombin do not produce normal release, in contrast to platelet secretion defects. Platelet aggregation testing is variable in α storage pool deficiency. ADP and epinephrine-induced aggregation are often normal. Collagen and thrombin-induced aggregation are more frequently abnormal. Platelet aggregation patterns in $\alpha\delta$ storage pool deficiency are similar to δ storage pool deficiency as the deficiency in δ granules appears more severe than the deficiency of α granules in these individuals. Platelet counts are normal in the storage pool deficiencies. Morphology is normal except in the *gray platelet syndrome* and Chédiak-Higashi syndrome.

Primary Secretion Defects (Enzymatic Pathway Defects) These congenital defects are rare and usually do not result in severe bleeding. Cyclo-oxygenase deficiency results in deficient conversion of membrane-associated arachidonic acid to thromboxane A_2 . Thromboxane synthetase deficiency and other defects of thromboxane A_2 generation and calcium mobilization have been described.³⁶

Treatment

Treatment of storage pool deficiencies and primary secretion defects has primarily been with judicious use of platelet transfusions for prevention or treatment of bleeding. For procedures with low risk of bleeding or where bleeding is easily controlled by local measures, no additional treatment may be needed. Prednisone has been reported to improve the bleeding

time but not platelet aggregation defects, presumably owing to an effect to promote vascular integrity. Thrombin-soaked Gelfoam may be used to control superficial cutaneous bleeding that does not respond to simple occlusive measures. For invasive trauma or surgical procedures, DDAVP can be used. Oral contraceptives may help to reduce menorrhagia. Recombinant factor VIIa has been reported to stop bleeding in an individual with Hermansky-Pudlak syndrome and menorrhagia. Red blood cell transfusion may also improve the platelet adhesion defect, possibly by supplying ADP. Avoidance of nonsteroidal anti-inflammatory agents or other drugs that induce platelet dysfunction is important in the management of these disorders.

Defects in Platelet Coagulant Activity

The bleeding pattern in these disorders is different from other platelet function defects since it involves an abnormality of coagulant activity. Easy bruising and epistaxis are not predominant features. Bleeding after surgery, tooth extractions, and childbirth occur. Spontaneous pelvic hematomas and hematomas after trauma are also seen. This pattern of bleeding more closely resembles the bleeding in hemophilias. (Refer to Chapter 27.)

Platelet function tests are normal in affected individuals, but the prothrombin time is abnormal. Specific assays for platelet factor 3 activity, which measures the platelet contribution to coagulation, should be abnormal.

Treatment for bleeding is usually with platelet transfusions to provide platelet procoagulant activity. Concentrates of prothrombin complex proteins have been used but have a risk of thrombosis, and therefore should be used only for serious bleeding problems.

von Willebrand Disease

von Willebrand disease is an inherited deficiency of vWF that may be confused with a primary platelet defect because of the similarities in clinical presentations between von Willebrand disease and platelet disorders. von Willebrand disease was originally termed *parahemophilia*, because it is a congenital bleeding disorder; however, it has a different inheritance pattern and different bleeding pattern than hemophilia.³⁷

vWF is a large, multimeric glycoprotein coded for by a gene located on chromosome 12 (Fig. 26-8). vWF is synthesized by vascular endothelial cells and stored in Weibel-Palade bodies. A small amount of vWF is also synthesized in megakaryocytes and stored in the platelet α granules. Polymerization of vWF occurs in endothelial cells with the production of high molecular weight multimers. vWF-cleaving protease (ADAMTS 13) reduces the size of the multimers at the time vWF is secreted into the plasma. Circulating vWF forms a complex with factor VIII, the protein that is deficient or defective in hemophilia A. vWF acts as a carrier protein for factor VIII, serving to protect the factor VIII molecule from proteolytic degradation and increase its concentration at the site of tissue injury. In normal individuals, plasma levels of factor VIII closely correlate with plasma levels of vWF.

The role vWF plays in hemostasis is quite important, as seen from the bleeding manifestations in patients with von

THE FACTOR VIII/VWF COMPLEX IN PLASMA

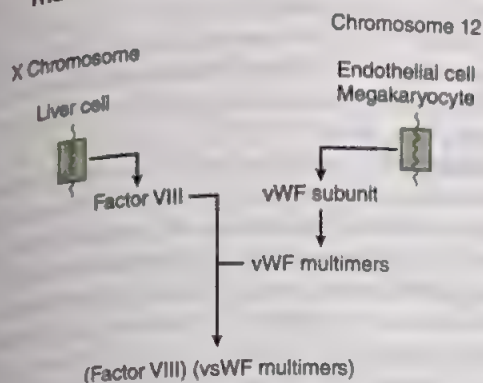


FIGURE 26-8 Factor VIII/von Willebrand factor (vWF) complex in plasma.

Willebrand disease. vWF is an important adhesive protein after vascular injury. It serves as a ligand between platelets, vascular endothelium, and other adhesive proteins such as fibronectin (Fig. 26-9). VWF has distinct domains for binding to platelet GPIb/IX/V, GPIIb/IIIa, and subendothelial components heparin and collagen. Platelet GPIb serves as the major receptor for vWF. Platelet adhesion is dependent on subendothelium, GPIb/IX/V, and vWF. In von Willebrand disease, the platelets that are intrinsically normal exhibit abnormal adhesion because of the absence or dysfunction of vWF. VWF promotes secondary hemostasis by functioning as a carrier for factor VIII. In von Willebrand disease, absence or dysfunction of vWF results in decreases in factor VIII and abnormal secondary hemostasis.

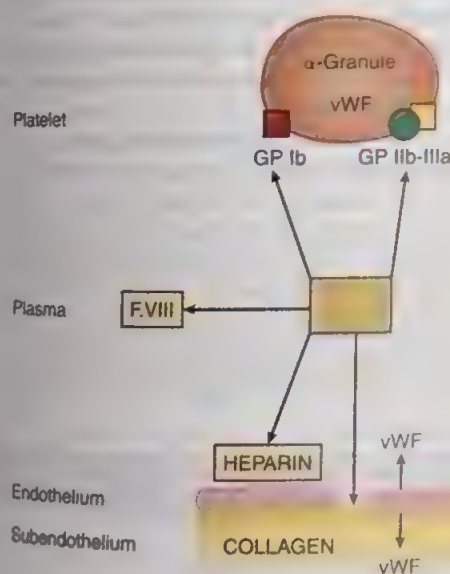


FIGURE 26-9 Schematic representation of the interactions between vWF, platelets, and collagen of the subendothelium. vWF synthesized by endothelial cells is released in plasma, stored in the granules of platelets and can be released after stimulation. vWF mediates platelet adhesion through binding to collagen and to platelet glycoprotein Ib (GPIb) in the presence of ristocetin, as well as to the platelet GPIIb/IIIa in the presence of physiological agonists (thrombin, collagen, ADP). vWF also binds to factor VIII (FVIII) and heparin.

Measurements of factor VIII activity, vWF antigen, and vWF activity are important in differentiating von Willebrand disease from hemophilia A and to define the types of von Willebrand disease.

von Willebrand disease comprises both quantitative and qualitative abnormalities of the large multimeric vWF glycoprotein. Affected individuals exhibit mucocutaneous bleeding typical of defects of primary hemostasis, in contrast to the joint and deep muscle bleeding typical for hemophilia. The disorder has an autosomal pattern of inheritance but has variable penetrance for expression in affected individuals. Severe cases are characterized by recurrent, potentially life-threatening bleeding, whereas mild cases may go undetected. Diagnosis of von Willebrand disease is often difficult because of the variability of abnormal laboratory results within affected individuals or family members. vWF and factor VIII are both acute phase reactant proteins, and plasma levels may be increased with stresses such as inflammation, surgery, and pregnancy or with estrogen therapy. Furthermore, the levels of vWF vary with ABO blood types, with the lowest levels being seen in individuals of O blood type.

Classification von Willebrand disease can be classified into three general categories:

1. Type 1, an autosomal-dominant disorder accounting for 70% of all cases, which is characterized by a quantitative decrease in normal vWF and mild bleeding
2. Type 2, which has variable inheritance with numerous subtypes, accounts for most of the remaining cases and is characterized by a qualitative abnormality in the structure of vWF
3. Type 3, a rare autosomal-recessive disorder characterized by absent levels of vWF multimers and a severe bleeding diathesis very similar in presentation to hemophilia A³⁹

von Willebrand Normandy (type 2N) is an unusual type 2 variant of von Willebrand disease that is characterized by a defect in the vWF binding to factor VIII. This disorder is sometimes confused with mild Hemophilia A because the factor VIII coagulant level is reduced but the vWF antigen and activity levels are normal or elevated. Clinically, the bleeding diathesis associated with von Willebrand "Normandy" is similar to other patients with type 2 von Willebrand disease.

A platelet-type von Willebrand disease has also been described. It is a platelet disorder characterized by increased binding of normal vWF to platelets caused by point mutations in platelet glycoprotein Ib α (GPIb α). The disorder also appears to be inherited in an autosomal dominant pattern. There is variable thrombocytopenia, possibly due to shortened platelet survival from this increased vWF binding to platelets. Platelet transfusions and von Willebrand factor concentrates are the most effective treatments.⁴⁰

Laboratory Evaluation

The routine laboratory evaluation of patients suspected of having von Willebrand disease includes a bleeding assessment tool, PFA-100 assay, platelet count, factor VIII coagulant activity, quantitative measurements of plasma vWF

antigen (vWF:Ag) by crossed immunoelectrophoresis or agarose gel electrophoresis, and functional assays of platelet-dependent vWF activity (ristocetin cofactor activity or the newer vWF:GPIb alpha binding assays).⁴¹ Subtypes of von Willebrand disease can be distinguished according to the vWF:Ag, vWF activity, factor VIII coagulant activity, and multimeric pattern on gel electrophoresis. All types of von Willebrand disease will have reduced vWF activity, but other laboratory findings will vary between the types. This may lead to uncertainty about the diagnosis, and genetic analysis may be useful to confirm a diagnosis. Platelet-type von Willebrand disease has reduced plasma vWF levels, reduced low molecular weight vWF multimers, and enhanced vWF binding similar to type 2 von Willebrand disease. Since the defect occurs on the platelet surface, vWF from affected individuals will bind normally to normal platelets, and vWF from normal individuals will bind abnormally to platelets of affected individuals.

Molecular testing may complement phenotypic laboratory testing. This technique is expensive and not widely available, which limits its use. It appears most helpful in diagnosis for differentiating type 2N from Hemophilia A and in differentiating type 2B and from platelet type vWD and in genetic counseling for type 3 vWD.^{39,42}

Treatment

For patients with type I von Willebrand disease, DDAVP is considered the initial treatment of choice. DDAVP stimulates the release of vWF from endothelial cell Weibel-Palade bodies. Baseline plasma vWF antigen and activity increase about three- to four-fold after DDAVP administration. vWF stores within the endothelial cells are depleted after about 3 to 4 days of DDAVP treatment. Patients requiring longer treatment or those who do not achieve an adequate response to DDAVP may require the use of intermediate purity factor VIII products such as Humate P, which contain intact vWF. Cryoprecipitate has been used in the past, but NIH guidelines suggest that cryoprecipitate only be used under rare circumstances to treat vWD, such as when potential exposure to infectious agents can be limited by using directed donations to prepare the product. The use of cryoprecipitate is strongly discouraged by the National Hemophilia Foundation, except in life- or limb-threatening situations when no vWF concentrate is available, because cryoprecipitate is not virally inactivated. In late 2015, a recombinant von Willebrand factor concentrate, Vonvendi, was approved for use in the United States. As it does not contain factor VIII coagulant protein, supplementation of the first dose with recombinant factor VIII coagulation factor concentrate is often required.

Patients with type 3 von Willebrand disease do not make vWF and do not respond to DDAVP. Therefore, they must be treated with intermediate purity factor VIII products or recombinant vWF with recombinant factor VIII.

There are many subtypes of type 2 von Willebrand disease, some of which respond to DDAVP and some of which do not. Thrombocytopenia may be present and worsen with DDAVP in the type 2B variant due to increased vWF binding.³⁹ In many of the type 2 variants, response to therapy is difficult to quantitate and may require measurement of PFA-100 with

determination of correction of platelet function after administration of appropriate therapy.

Antifibrinolytic agents can be utilized as an adjunctive therapy in all patients with von Willebrand disease. For dental procedures, local measures are used, and replacement therapy might not be necessary.

Treatment of platelet-type von Willebrand disease with measures to increase plasma vWF can actually cause thrombocytopenia by inducing clearance of platelets from the circulation. For minor bleeding, antifibrinolytic therapy may suffice. DDAVP can be considered if a prior trial demonstrating effectiveness in raising vWF ristocetin cofactor activity and safety regarding a decrease in platelet count response has been demonstrated for the patient. Platelet transfusions if the thrombocytopenia is severe and vWF-rich concentrates if vWF is low are the most effective treatments.

Acquired Qualitative Platelet Disorders

Platelet dysfunction can also be caused by many systemic illnesses and medications (Box 26-4). In these diseases, the platelet dysfunction is a secondary manifestation and not typically one of the primary presenting features of these disorders. Acquired causes of platelet dysfunction are more commonly encountered than congenital platelet disorders; however, the results of platelet function tests can appear similar to those seen with congenital disorders. To differentiate between acquired and congenital causes of platelet dysfunction, a careful history and physical examination as well as family history must be obtained.

Uremia

Bleeding and thrombosis are recognized complications of uremia. Patients with acute and chronic renal failure generally exhibit bleeding from mucous membranes.⁴³ Petechiae, purpura, epistaxis, ecchymosis, and gastrointestinal bleeding are common. Severe hemorrhage within serous cavities and muscles may also occur.

A number of different laboratory findings and clinical symptoms suggest that platelet dysfunction with abnormal platelet-vessel wall interaction is the major cause of platelet dysfunction and hemorrhage. Studies have shown an abnormality in the interaction of vWF and platelet GPIIb/IIIa complex in uremic patients; however, platelet membrane glycoproteins Ib, IIb, and IIIa are quantitatively normal. Other platelet abnormalities seen in uremia include abnormal prostaglandin synthesis, decreased membrane procoagulant activity, decreased

BOX 26-4 Acquired Qualitative Platelet Disorders

- Renal disease (uremia)
- Liver disease
- Paraproteinemias
- Myeloproliferative disorders
- Acquired von Willebrand disease
- Cardiopulmonary bypass
- Acquired storage pool deficiencies
- Drug therapy

platelet serotonin release, abnormal β -thromboglobulin levels, elevated intracellular calcium, and decreased thromboxane synthesis. Increased levels of "uremic toxins," such as guanidosuccinic acid and phenols, are rather consistent findings in patients with uremia. The acquired platelet defects associated with uremia are thought to be primarily mediated by these products. A correlation of the metabolic changes with the exact mechanism of the functional defect has not been firmly established. There may also be a defect in platelet activation since platelets in some uremic individuals demonstrate reduced fibrinogen binding, secretion, and aggregation responses.

Mild thrombocytopenia with platelet counts as low as $100 \times 10^9/L$, decreased adhesion, and abnormal aggregation are abnormal laboratory findings often seen in patients with uremia. Drug interactions with platelets affecting platelet function may also contribute. Activation of platelets from the dialysis filter may also promote bleeding in individuals on dialysis. Platelet aggregation studies in uremic patients typically show no characteristic patterns.

The anemia of renal failure may contribute to the hemostatic defects observed in uremic patients. Red blood cells may play a role in hemostasis by improving factor VIII coagulant function and by providing ADP, a platelet-aggregating agent. EPO (r-HuEPO) therapy has proved to be effective in improving hemostasis and decreasing the bleeding tendencies in uremic patients on dialysis.

Hemodialysis or peritoneal dialysis is the treatment of choice to correct the hemostatic defect in uremia. However, platelet function abnormalities may remain. Administration of cryoprecipitate to patients unresponsive to dialysis has been used to correct acute bleeding. The use of DDAVP in uremic patients has also prevented clinical bleeding in patients after surgical procedures and has shortened the prolonged bleeding time. Conjugated estrogens have been reported to shorten the bleeding time in uremia and limit bleeding in patients who develop gastrointestinal telangiectasias. The mechanism of action of these therapeutic measures is not clear.

Liver Disease

Chronic liver disease is often associated with a significant hemorrhagic diathesis as a result of multiple alterations in hemostasis, including platelet dysfunction. Mild to moderate thrombocytopenia is seen in approximately one-third of patients with chronic liver disease as a result of splenic sequestration secondary to congestive splenomegaly associated with portal hypertension. Abnormal platelet function tests found in patients with chronic liver disease include reduced platelet adhesion; abnormal platelet aggregation to ADP, epinephrine, and thrombin; and abnormal PF3 availability. An acquired storage pool deficiency has also been suggested. The exact mechanism responsible for the platelet defects seen in chronic liver disease is not known.

Treatment of bleeding in a patient with chronic liver disease may require several modalities simultaneously to correct the multiple abnormalities present. Transfusion with platelet concentrates may ameliorate the bleeding and thrombocytopenia associated with chronic liver disease; however, the

expected rise in platelet count following a transfusion may be blunted if splenic sequestration or increased platelet consumption occurs. Avatrombopag was originally approved for clinical use to increase platelet counts in patients with cirrhosis before surgical procedures. DDAVP may improve the qualitative platelet defect associated with this disorder. The numerous coagulation factor deficiencies common in chronic liver disease typically require the administration of fresh frozen plasma to correct the coagulation abnormalities. Recombinant factor VIIa is associated with a thrombotic risk and is not used in this situation. Use of conjugated estrogens may decrease the overall bleeding tendency after an acute episode.

Paraproteinemias

Patients with lymphoproliferative malignancies may exhibit both hemorrhagic diatheses and hypercoagulability. These hemostatic alterations are complex and multifactorial. Clinically significant bleeding is a manifestation associated with multiple myeloma, Waldenström's macroglobulinemia, and other related malignant paraprotein disorders. The pathogenesis of hemorrhage and other recognized hemostatic abnormalities has been proposed to be caused by the interaction of the paraprotein with platelets and coagulation factors. Clinical bleeding and platelet dysfunction are seen in approximately 60% of patients with IgM monoclonal gammopathy (Waldenström's macroglobulinemia) compared with approximately 40% of the patients with IgA myeloma and 15% of patients with IgG myeloma.

The hemorrhagic diathesis of paraproteinemias correlates with the impairment of platelet function. Thrombocytopenia and inhibition of coagulation factors may also contribute to the bleeding tendency. Patients may present clinically with spontaneous epistaxis and ecchymosis, or unexplained postoperative bleeding in the face of a normal platelet count and coagulation profile. Patients with malignant paraprotein disorders should not ingest aspirin or aspirin-containing drugs because this may exacerbate the platelet defect. Uremic platelet dysfunction associated with renal failure often seen in the malignant paraproteinemias may also contribute to the hemorrhagic episodes.

Platelet abnormalities include decreased aggregation in response to various aggregating agents, altered shape change, and abnormal release reactions. Acquired von Willebrand syndrome causing reduced levels of vWF/factor VIII complexes, other circulating anticoagulants, amyloid-associated coagulopathies, impaired fibrin formation and polymerization, hyperviscosity syndrome, nephrosis, and DIC represent additional abnormalities that predispose patients with paraproteinemia to bleeding.

Thrombocytopenia, unrelated to the paraprotein effect, may result from marrow replacement, chemotherapy, or hypersplenism and is a common finding contributing to the incidence of significant bleeding.

Therapy for patients with qualitative defects resulting from multiple myeloma and related disorders includes plasmapheresis to reduce the circulating paraprotein concentrations and chemotherapy to inhibit paraprotein production.

Blood product support and other therapies may be required when additional hemostatic abnormalities are present.

Myeloproliferative Neoplasms

The MPNs include polycythemia vera (PV), primary myelofibrosis (MF), chronic myelogenous leukemia (CML), and essential thrombocythemia (ET) (see Chapters 18 and 19). This group of disorders has many clinical and hematologic features in common, but the hemostatic and thrombotic problems are variable. The hemorrhagic manifestations include ecchymosis, epistaxis, and mucocutaneous bleeding from the gastrointestinal and genitourinary tracts. Thrombosis occurs in both the arterial and venous circulation and includes deep vein thrombosis, pulmonary embolism, stroke, myocardial infarction, and thrombosis of the hepatic, portal, splenic, and mesenteric veins. Patients may present with bleeding and thrombosis simultaneously or alternate with bleeding and thrombotic episodes as the disease progresses. Intrinsic platelet function defects are noted frequently and may contribute to a bleeding tendency. In many cases, thrombocytosis is a contributing factor, especially for thrombotic problems in patients with an MPN. Older age, cardiovascular risk factors, previous thromboses, and presence of a JAK2 V617F mutation are risk factors for thrombosis.⁴⁴

Aggregation patterns in these disorders are usually not characteristic. The most consistently noted laboratory abnormalities include abnormal release and aggregation in response to epinephrine, collagen, and ADP, but these findings vary from patient to patient with the same type of MPN. Other reported abnormalities include acquired storage pool defects, abnormal prostaglandin and arachidonic acid metabolism, and platelet hyperactivity.

Thrombosis is a major complication of PV owing to an increased red blood cell mass with increased whole blood viscosity and thrombocytosis. The JAK2 V617F mutation appears to have an effect on both platelets and nonhematopoietic cells to promote thrombosis.⁴⁵ Thrombocytosis is generally moderate, in the range of $500 \times 10^9/L$ to $1 \times 10^{10}/L$, and is seen in approximately 50% to 60% of cases. Phlebotomy as treatment to limit erythropoiesis with development of iron deficiency may worsen the thrombocytosis. Bleeding less commonly occurs and is often due to peptic ulcer disease due to histamine from associated basophilia. Platelet function defects occur in approximately 50% to 60% of cases and may contribute to the bleeding risk. Abnormal platelet aggregation in response to epinephrine, ADP, and collagen has been reported in PV. Platelet activation and intravascular coagulation with increased plasma levels of β -thromboglobulin, low platelet serotonin levels, and abnormal levels of fibrinogen and prothrombin have also been reported in cases of PV.

Patients with MF may experience bleeding, such as ecchymosis and urogenital hemorrhage. Thromboembolic complications, with the exception of hepatic and portal vein thrombosis, occur rather infrequently. It has been suggested that of all the MPNs, MF has the greatest degree of platelet function defects. Decreased platelet adhesion is a relatively common finding in patients with MF. Storage pool deficiencies and impaired platelet aggregation have also been reported.

ET presents with thrombocytosis and is associated with both bleeding and thrombosis, although bleeding episodes occur more often. Evidence suggests that thrombocytosis alone does not account for the bleeding and thrombotic episodes seen in ET, but the combination of thrombocytosis and abnormal platelet function determine the hemostatic defects in ET. Decreased platelet aggregation in response to epinephrine as well as decreased platelet retention are noted in patients with ET. Impaired PF3 availability and platelet hyperactivity have been reported in patients with thrombosis in ET. Several platelet defects have been described in ET, but precisely what predisposes an individual patient to bleeding or thrombotic manifestations is still uncertain.

Bleeding and thrombosis occur least frequently in patients with CML. When present, bleeding includes mucocutaneous hemorrhages, retinal hemorrhages, and hematuria. It is thought that acquired platelet defects may be responsible for this bleeding as a result of dysplastic platelet production. Thrombocytopenia, as occurs with transformation to blast crisis, also results in a bleeding tendency. Decreased or absent platelet aggregation in response to ADP, epinephrine, and collagen have been reported in cases of CML. Factor V deficiency has been reported in some cases of CML. Increases in platelet vWF have also been noted.

Reduction of the risk of hemorrhage and thrombosis is a principal therapeutic goal in treatment of the MPNs. Cytoreductive chemotherapy to control erythrocytosis and thrombocytosis reduces these risks and helps control symptoms. In patients with marked thrombocytosis and cerebral ischemia, plateletpheresis in addition to chemotherapy may be helpful. Aspirin therapy is useful to prevent thrombosis in PV and ET or to treat a specific complication of pain and erythema of the hands termed *erythromelalgia* but may worsen a preexisting bleeding tendency. Hemorrhage may require administration of platelets to replace endogenous dysfunctional platelets.

Acquired von Willebrand Disease

Acquired von Willebrand disease is a rare bleeding disorder that has been found in patients with myeloproliferative disorders, lymphoproliferative diseases, monoclonal gammopathies, autoimmune disorders, and cardiovascular disorders.⁴⁶ Most patients are older, with no previous history of bleeding. Bleeding presents with an insidious onset and manifests as mucocutaneous or posttraumatic hemorrhage.

Pathologically, this disorder is caused by production of an antibody that specifically interacts with vWF. Acquired von Willebrand disease is caused by a diverse group of disorders, and the associated antibodies are a heterogeneous group that interact differently with the vWF protein from patient to patient. Because these antibodies are so varied, the laboratory findings seen in acquired von Willebrand disease are also variable from patient to patient but may include decreased plasma levels of factor VIII coagulant activity, vWF:Ag, and vWF activity, and abnormalities in vWF multimeric patterns. Platelet vWF is generally normal. No single test is sufficient to establish or exclude this disorder. The typical rise in factor VIII coagulant activity and vWF activity after infusion

of cryoprecipitate seen in congenital von Willebrand disease is not seen in the acquired form because of neutralization of these activities by antibody.

Spontaneous remission or remission after therapy for the underlying disease is typical of this disorder. Therapeutic use of DDAVP has been observed to correct the bleeding time and increase the level of vWF. vWF-containing concentrates, and increase the level of vWF. vWF-containing concentrates, recombinant Factor VIIa, intravenous gamma globulin, antifibrinolytics, and plasmapheresis to remove antibodies are used to treat acute bleeding. Treatment of the underlying cause is necessary for long-term control.

Cardiopulmonary Bypass

In the United States, approximately 400,000 patients per year undergo cardiopulmonary bypass surgery. Alterations in hemostasis and life-threatening hemorrhage have been documented. Various hemostatic abnormalities are implicated in the development of the hemorrhagic manifestations of cardiopulmonary bypass surgery. These abnormalities include decreases in platelet number and function, factor deficiencies resulting from consumption and hemodilution, increased fibrinolytic activity, DIC, and inadequate or excess neutralization of heparin with protamine.⁴⁷ Platelet activation and platelet dysfunction account for the majority of bleeding complications seen during this procedure. Proposed mechanisms include platelet activation with granule release during exposure to the extracorporeal unit and plasmin degradation of platelet membrane receptors. Post-bypass diffuse microvascular bleeding occurs with greater frequency in patients undergoing repeat surgery or complicated cardiac procedures compared with patients undergoing surgery for the first time.

Platelet concentrates are administered to stop bleeding. Fresh frozen plasma and cryoprecipitate should be given only to treat bleeding associated with coagulation factor deficiencies. DDAVP is often given prophylactically for high-risk patients or to stop bleeding after it occurs. Antifibrinolytic agents may also help to minimize postoperative blood loss.

Acquired Storage Pool Deficiencies

Acquired storage pool deficiencies have been reported in patients with SLE, ITP, TTP, DIC, HUS, MPNs, hairy cell leukemia, acute nonlymphocytic leukemia, chronic lymphocytic leukemia, and in cardiopulmonary bypass. The acquired platelet defect in each disorder may be variable and can be caused by either production of dysfunctional platelets (i.e., MPNs) or depletion of storage pool constituents resulting from injury or activation (DIC).

Drug Therapy

A large variety of pharmacological drugs may affect platelet function (Box 26-5). Some drugs may induce thrombocytopenia. Other drugs alter platelet responses. Drug-induced alterations of hemostasis that cause activation of platelet function clinically manifest as thrombosis, whereas drugs that inhibit platelet function clinically manifest as hemorrhage. Individual susceptibility to the effects of these drugs varies greatly. Most often, the acquired drug-induced hemostatic abnormalities observed are transitory and disappear when the drug is

BOX 26-5 Drugs That Can Inhibit Platelet Function

- Aspirin
- Sulfinpyrazone
- Dipyridamole
- Clopidogrel, Prasugrel, Ticagrelor
- Antimicrobials
 - Penicillins
 - Cephalosporins
- Dextran
- GP IIb/IIIa inhibitors
 - Abciximab
 - Tirofiban
 - Eptifibatide

discontinued. However, other effects, such as those seen in HITTS, may be irreversible or disappear more slowly.

Drugs that inhibit platelet function do so by a variety of mechanisms that include altering prostaglandin synthesis, phosphodiesterase activity, platelet membrane or membrane receptors, and cyclic adenosine monophosphate (cAMP) levels. However, the mechanism of platelet inhibition for many drugs is still uncertain.

Aspirin has been well documented to inhibit platelet aggregation and platelet secretion in response to ADP, epinephrine, and low concentrations of collagen. Aspirin inhibits prostaglandin synthesis by irreversible acetylation and inactivation of cyclo-oxygenase (COX-1), thereby inhibiting endoperoxide and thromboxane A_2 synthesis, which are two important mediators of platelet release. The inhibitory effect of aspirin on thromboxane A_2 synthesis remains for the life span of the platelet. Aspirin also inhibits endothelial cell synthesis of prostacyclin; however, inhibitory action is predominantly against platelet cyclo-oxygenase. Low-dose aspirin treatment takes advantage of this differential effect on vascular and platelet cyclo-oxygenase. Patients with hemophilia, von Willebrand disease, or major underlying hemostatic defects may develop significant or spontaneous bleeding after aspirin ingestion and should avoid aspirin or aspirin-containing compounds. Salicylate and choline magnesium trisalicylate do not contain an acetyl group and do not inhibit cyclo-oxygenase. These drugs are preferable in patients with underlying hemostatic disorders.

Certain other nonsteroidal anti-inflammatory agents (NSAIDs), such as indomethacin, ibuprofen, phenylbutazone, and naproxen, can induce significant platelet dysfunction by inhibiting prostaglandin synthesis. These drugs inhibit cyclo-oxygenase, but the effect is reversible. The selective COX-2 inhibitor celecoxib does not affect platelet function but still has been associated with gastrointestinal bleeding due to its COX-2 effect.

Other drugs suppress platelet function by inhibiting cAMP production. cAMP plays a major role in mediating platelet activity. Platelet response is inhibited when intracellular levels of cAMP are raised. Drugs such as dipyridamole inhibit phosphodiesterase, an enzyme capable of inactivating cAMP, resulting in increased cAMP levels in platelets. Dipyridamole

has a mild effect on platelet function by itself and does not prolong the bleeding time; however, when administered in a time release form with aspirin (Aggrenox), it exerts a greater antiplatelet effect.

Clopidogrel, prasugrel, and ticagrelor are utilized in patients undergoing cardiac catheterization and coronary stent placement and in patients with transient ischemic attacks or completed strokes to inhibit platelet function. Their mechanism of action is inhibition of platelet aggregation by binding to platelet surface P2Y₁₂ ADP receptors to reduce platelet-fibrinogen binding. This effect is irreversible and lasts the life of the affected platelet. These drugs are often used in conjunction with aspirin therapy to induce two separate mechanisms of platelet inhibition. When drugs such as these with differing mechanisms are utilized together, the hemorrhagic complications may increase.

Antimicrobial drugs such as penicillin, ampicillin, and carbenicillin may inhibit platelet function through interference with membrane or membrane receptors. When administered in high doses, bleeding may occur. Cephalosporin antibiotics may also inhibit platelet function by affecting platelet aggregation, platelet secretion, and platelet adhesion.

Dextran also impairs platelet function and may cause bleeding. Platelet function tests such as aggregation, PF3 availability, and platelet retention have all been shown to be abnormal with the use of dextran. It is believed that dextran interferes with the platelet surface membrane to cause platelet dysfunction.

Several GPIIb/IIIa receptor antagonists have been developed and approved for intravenous human use in acute coronary syndromes or for percutaneous coronary interventions, such as angioplasty or stent placement, to prevent reocclusion. Abciximab (Reopro) is a mouse-human chimeric Fab fragment that exhibits irreversible, noncompetitive binding to platelets. Recovery of platelet function is noted by 48 hours after administration, but platelet-bound antibody is still detectable for 14 days after administration. Eptifibatide (Integrilin) is a cyclic heptapeptide that contains a lysine-glycine-aspartic acid (KGD) sequence similar to that found on adhesive proteins such as fibrinogen or vWF. This peptide has reversible, competitive binding to platelets, with recovery of platelet function 2 to 4 hours after administration. Tirofiban (Aggrastat) is a nonpeptide GPIIb/IIIa inhibitor that also exhibits reversible, competitive binding to platelets with a similar recovery of platelet function in 2 to 4 hours.

Bleeding is a potential complication of all of these anti-thrombotic drug treatments and is often noted when excessive doses of heparin are used concomitantly with these drugs. Heparin dosing based on body weight has reduced the risk of serious and fatal bleeding complications. Thrombocytopenia, which may also occur with the GP IIb/IIIa receptor antagonists, as noted in the previous discussion of drug-induced thrombocytopenias, is a less frequent complication but may be severe (less than $10 \times 10^9/L$) and contribute to the bleeding tendency with use of these drugs. Onset of the thrombocytopenia is rapid, usually within 12 to 24 hours of treatment. Thrombocytopenia caused by these agents is often mistaken for heparin-induced thrombocytopenia because patients

typically are given both agents concomitantly. The precipitous onset of thrombocytopenia after initiation of therapy may be helpful in differentiating GPIIb/IIIa inhibitor-induced thrombocytopenia from heparin-induced thrombocytopenia. Thrombocytopenia appears to occur more frequently with abciximab than with eptifibatide or tirofiban.

Numerous other conditions have been associated with an acquired platelet defect. These include paroxysmal nocturnal hemoglobinuria, infectious mononucleosis, severe vitamin B₁₂ deficiency, diabetes, hyperbetalipoproteinemia, congenital heart defects, and hypothyroidism. The pathogenesis in these various disorders is uncertain.

Vascular Disorders

Purpura can be caused by abnormalities of skin, connective tissue, or blood vessels, or may be a result of inflammatory processes that are not associated with quantitative or qualitative platelet disorders. These may be congenital or acquired vascular disorders and are listed in Box 26-6.

Primary Purpura

Primary purpura comprises disorders that result in bruising but are not associated with any specific disease. Simple purpura or "devil pinches" occur as a result of increased skin fragility. The bruising is usually mild and occurs with minimal trauma. Often, there may be a family history of easy bruising. Mechanical purpura occurs as a result of sudden increases in capillary pressure and usually manifests as petechiae. Sneezing, coughing, Valsalva maneuvers, or seizures may cause this problem. Actinic (senile) purpura, which is seen in older individuals, is similar to that occurring in individuals who are receiving corticosteroid therapy. In these disorders, the purpuric lesions usually occur on the hands and arms and result from loss of subcutaneous tissue and support of blood vessels in affected skin. Factitious purpura is caused by self-induced trauma and usually is found on areas of the body that are easily accessible. The purpura may be caused by pinching, suction applied to the skin, or a blow to the skin. An entity termed psychogenic purpura has been described that is seen in individuals with emotional problems, often after severe trauma or extensive surgery, which may be a hypersensitivity reaction to red blood cell membrane components or DNA hypersensitivity. Various skin disorders such as Schamberg's purpura (progressive pigmentary dermatosis) and related conditions result in purpuric lesions. These lesions are usually seen bilaterally on both lower legs and appear as a result of a noninflammatory disorder of capillaries of the skin. If the disorder is long-standing, hemosiderin pigmentation is noted in previously involved areas.

Secondary Purpura

Secondary purpura results from another disease process and is just one of the manifestations of that disease process. These disorders are acquired and have no associated family history.

Infectious Purpura

Purpura may result from various infectious diseases. Bacterial infections such as meningococcemia, diphtheria, streptococcal or staphylococcal bacteremia, and rickettsial infection such as Rocky Mountain spotted fever or parasitic

BOX 26-6 Vascular Purpuras**Primary Purpura**

- Simple purpura
- Mechanical purpura
- Senile purpura
- Factitious purpura
- Schamberg's purpura

Secondary Purpura

- Infectious purpura
 - Waterhouse-Friderichsen syndrome
 - Purpura fulminans
 - Septic emboli
- Allergic purpura
- Schönlein-Henoch purpura
- Drug sensitivity

Metabolic Purpura

- Scurvy
- Cushing's syndrome
- Diabetes mellitus
- Protein C deficiency

Psychogenic Purpura

- Gardner-Diamond syndrome
- DNA hypersensitivity

Purpura Secondary to Dysproteinemia

- Waldenström's purpura
- Cryoglobulinemia
- Amyloidosis
- Hyperviscosity syndrome

Vascular and Connective Tissue Disorders

- Hereditary hemorrhagic telangiectasia
- Angiodysplasia
- Giant hemangiomas (Kasabach-Merritt syndrome)
- Ehlers-Danlos syndrome
- Marfan syndrome
- Pseudoxanthoma elasticum
- Osteogenesis imperfecta

infection by malaria may cause purpura. The purpura may be caused by direct damage to blood vessels by the particular organism (vasculitis) or result from the effects of endotoxin on blood vessels. Septic emboli to the skin (ecthyma gangrenosum) may be seen in endocarditis. In the Waterhouse-Friderichsen syndrome, meningococcemia results in adrenal hemorrhage with adrenal insufficiency and shock. Purpura fulminans is an acute purpuric syndrome that usually occurs during septic shock, with development of ischemia followed by thrombosis and necrosis of affected areas, often the fingers and toes.⁴⁸ Meningococcemia, streptococcal infections, and viral infections may provoke this life-threatening problem. If the individual survives, necrosis of the fingers and toes may require amputation. DIC is often seen with Waterhouse-Friderichsen syndrome or purpura fulminans.

Heparin may be used when large vessel thrombosis or central venous catheter thrombosis occur. Warfarin should be avoided due to its effect on Protein C and S production. Activated Protein C (Drotrecogin alpha, Xigris) concentrate and hyperbaric oxygen have been used to treat this disorder.

Allergic Purpura

Allergic purpura is the result of an allergic vasculitis. The purpuric lesions are usually palpable due to their inflammatory nature. Schönlein-Henoch purpura is a vasculitis involving the skin, gastrointestinal tract, kidneys, heart, and central nervous system.⁴⁹ This syndrome is considered an immune complex disease and is characterized by involvement of capillaries with a diffuse perivascular infiltration by neutrophils, lymphocytes, and macrophages. Some patients have IgA deposition in the kidney and elevated serum IgA levels. This syndrome may be related to IgA nephropathy whereby similar IgA deposition in the kidney is noted. In Schönlein-Henoch purpura, the onset is abrupt, with a macular rash (usually symmetric) involving the lower extremities that becomes purpuric. In some individuals, the joint and gastrointestinal symptoms predominate, and the cutaneous expression is minimal. This disorder is seen most commonly in children. Renal dysfunction is common and typically reversible in children. Overt renal failure is most common in affected adults. Schönlein-Henoch purpura may last several weeks and then recur (Fig. 26-10).

Drug hypersensitivity may also result in allergic purpura. This must be differentiated from drug-induced thrombocytopenias that result in purpura. The mechanism by which these drugs cause purpura is not understood. Aspirin, atropine, iodides, penicillin, quinine, and sulfonamides have been described to cause allergic hypersensitivity purpura.

Metabolic Purpura

Metabolic purpura is caused by hormonal or biochemical abnormalities. Scurvy is caused by a deficiency of vitamin C. This was a common problem several centuries ago in sailors who did not have access to vitamin C on long voyages. In modern times, this disorder is found in refugees from war or famine, adults with inadequate or fad diets, and in alcoholics. Deficiency of vitamin C appears to cause a defect in the



FIGURE 26-10 Anaphylactoid (Schönlein-Henoch) purpura. Purpuric lesions of the foot.

synthesis of collagen in the walls of small blood vessels. Vitamin C is a cofactor for hydroxylation of proline and lysine in the production of collagen and keratin. A platelet function defect may also be present, with a decrease in the platelet adhesion reaction. Scurvy may present with leg swelling, pain, or discoloration. Subperiosteal bone bleeding is characteristic of scurvy and may be noted radiologically. Gingival bleeding and hemarthrosis may also be present. Perifollicular hemorrhages with hyperkeratosis of the hair follicles and "corkscrew" hairs are characteristic of this problem. Vitamin C administration orally cures the disorder.

Cushing's syndrome caused by corticosteroid excess results in purpura (Fig. 26-11). The pathogenesis is identical to that seen with steroid or purpura, including atrophy of the subcutaneous tissue and increased blood vessel fragility. Other cutaneous stigmata of Cushing's syndrome include a "moon" face, pigmented abdominal striae, a "buffalo hump" on the lower neck and upper back, with wasting of the extremities and an obese abdomen.

Diabetes mellitus may be associated with retinal hemorrhages. This is a result of vascular proliferation caused by retinal hypoxia. These fragile vessels may leak fluid or hemorrhage because of their increased permeability and can result in retinal detachments. Laser photocoagulation and optimal control of hyperglycemia are treatments used for this problem. No endogenous platelet or coagulation disorder is present in this situation. Similarly, sickle cell anemia and related disorders may also cause retinal hemorrhages by a similar mechanism and are treated with laser photocoagulation.

Protein C deficiency may occur on a congenital or acquired basis and is a risk factor for thrombosis.⁵⁰ An unusual syndrome that resembles purpura fulminans can be present in neonates, resulting from inherited homozygous protein C deficiency. This disorder presents hours after birth and is usually lethal. Protein C deficiency also results from oral anticoagulant therapy because protein C is a vitamin K-dependent protein. Rapid depletion of protein C by warfarin appears to cause a unique thrombotic disorder termed *warfarin skin necrosis syndrome*. This unusual disorder presents as painful, erythematous areas on the buttocks, breasts, legs, or penis,



FIGURE 26-11 Steroid purpura (skin manifestations).

which rapidly turn to hemorrhagic bullae and then become necrotic. Cessation of warfarin and administration of plasma that contains protein C or activated protein C concentrate are appropriate treatments. Vitamin K administration may be helpful but will usually not reverse the disorder soon enough to prevent necrosis.

Purpura Secondary to Dysproteinemia

Purpura caused by dysproteinemias encompasses several disorders. Benign hypergammaglobulinemic purpura (Waldenström's purpura) is a disorder of women that presents with recurrent purpura on the lower extremities and resultant hemosiderin staining of the skin similar to Schamberg's purpura.⁵¹ Unlike Schamberg's purpura, polyclonal hypergammaglobulinemia and an elevated erythrocyte sedimentation rate are noted. Mild anemia may be present. Purpura are made worse by leg dependency or wearing constrictive garments and improved with leg elevation and support stockings (Fig. 26-12). Skin biopsy reveals necrotizing vasculitis. Patients may develop Sjögren's syndrome or keratoconjunctivitis sicca, suggesting this may be a vasculitis that results from a connective tissue disorder.

Cryoglobulinemia is caused by production of cryoprecipitable serum proteins or protein complexes. This may result from primary plasma cell dyscrasias or from chronic viral infections such as hepatitis C. Exposure to cold may cause purpura on the skin of the extremities or face. The purpura may blister or ulcerate. The proposed pathogenesis for the purpura in the cryoglobulinemic disorders is vascular damage owing to precipitation of cryoglobulins.

Amyloidosis may cause purpura as a result of several mechanisms. Easy bruising is common in amyloidosis, with "pinch"



FIGURE 26-12 Typical lower extremity vascular lesions seen in patients with paraproteinemia.

purpura and ecchymosis from skin pressure during shaving. Postproctoscopic purpura occurs when a patient with amyloidosis is placed in a head-down position for proctoscopic examination, with characteristic purpura around the eyelids (Fig. 26-13). Bleeding is caused by deposition of amyloid protein around small blood vessels, resulting in vessel fragility. Low factor X levels from binding of factor X to amyloid fibrils, hyperfibrinolysis related to excessive fibrinolytic activity, and platelet function alterations may enhance the bleeding tendency. Treatment for bleeding caused by amyloidosis involves treatment of the underlying disease with chemotherapy. Splenectomy has also been used. Administration of plasma and platelet transfusions are not usually effective. Antifibrinolytic therapy, especially if hyperfibrinolysis is present, and recombinant Factor VIIa may be useful in controlling bleeding.

Hyperviscosity syndrome results from hypergammaglobulinemia owing to an increase in plasma viscosity. Blood flow is restricted because of hyperviscosity involving development of ischemia and vascular injury, with resultant hemorrhage. Purpura, gingival hemorrhages, and retinal hemorrhages are common. Central nervous system bleeding may be lethal. This disorder is usually seen in IgM syndromes such as Waldenström's macroglobulinemia, and IgA or IgG3 multiple myeloma. (Refer to Chapter 23.) It is rarely seen in multiple myeloma with other IgG subtypes. Treatment is with plasmapheresis to remove excessive IgM or IgG, and chemotherapeutic treatment of the underlying plasma cell dyscrasia to reduce paraprotein production.

Vascular and Connective Tissue Disorders

These disorders result from abnormal blood vessel structure, with purpura and bleeding caused by increased blood vessel permeability. Most of these disorders are congenital.

Hereditary Hemorrhagic Telangiectasia

Hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu syndrome) is caused by the development of telangiectasias of mucous membranes and skin (Fig. 26-14). This is an autosomal-dominant disorder that usually becomes obvious in adolescence or early adulthood.⁵¹ Lesions develop on the tongue, lips, palate, face, and hands. Similar lesions are present



FIGURE 26-14 Tongue of a patient with hereditary hemorrhagic telangiectasia. Note the multiple vascular telangiectases, which can occur in the nares, the oral mucous membranes, and throughout the gastrointestinal tract. Recurrent bleeding requiring transfusions is a common manifestation.

in nasal mucosa and throughout the gastrointestinal tract. The lesions blanch when pressure is applied, in contrast to petechiae, because these are vascular structures, not small hemorrhages. Chronic epistaxis and gastrointestinal bleeding occur and usually worsen as the patient grows older. Iron deficiency anemia is usual. The nasal bleeding can be difficult to control but may be treated with nasal packing, cauterization, or laser photocoagulation. Systemic or topical use of tranexamic acid may control epistaxis acutely. Topical thrombin spray may also help to control epistaxis. Administration of parenteral iron or red blood cell transfusions is usually necessary to treat the anemia. Estrogens, the estrogen receptor antagonist tamoxifen, and danazol have been reported to help in individual patients. More recently, it has been shown that patients with the disorder have elevated levels of plasma vascular endothelial growth factor (VEGF) and transforming growth factor-beta (TGF- β) secondary to mutations in the TGF- β signaling pathway.⁵² These mutations disrupt vascular integrity and smooth muscle differentiation resulting in fragile blood vessels. VEGF is an angiogenesis factor that promotes endothelial cell proliferation, leading to the AV malformations and telangiectasias present in the disorder. Bevacizumab, a recombinant monoclonal antibody that inhibits VEGF, appears effective in reducing bleeding and in reducing high cardiac output due to the shunting from the vascular malformations. Another antiangiogenesis treatment used as therapy for multiple myeloma is being evaluated in clinical trials to reduce vascular lesions and improve bleeding.

Angiodysplasia

Angiodysplasia is a disorder involving blood vessels of the gastrointestinal tract. The cutaneous lesions of hereditary hemorrhagic telangiectasia are not present. Vascular permeability leads to chronic gastrointestinal bleeding, anemia, and iron deficiency. This disorder has been associated with von Willebrand disease and may be caused by the effect of vWF on vascular structures. An acquired form of angiodysplasia has been reported in patients with aortic stenosis. In these patients,



FIGURE 26-13 Amyloid purpura. Note characteristic periorbital distribution.

aortic valve surgery has improved the bleeding. Hemicolectomy has been used to treat the gastrointestinal bleeding.

Giant Hemangiomas (Kasabach–Merritt Syndrome)

Giant hemangiomas may occur congenitally soon after birth and affect the skin, liver, and spleen. Unless these lesions spontaneously regress, DIC may develop. These hemangiomas have numerous thin-walled blood vessels that produce thromboplastic substances to incite DIC. Treatment is usually surgical removal or radiation if surgery is not possible. Interferon has been used in a single case report.

Congenital Connective Tissue Disorders

Several rare congenital disorders of connective tissue proteins have been noted to cause purpura and bleeding. Ehlers–Danlos syndrome is an autosomal-dominant disorder characterized by hyperdistensible joints and fragile skin. Thirteen different subtypes of the disorder are described related to abnormal collagen synthesis.⁵³ Hematomas, mucosal bleeding after dental procedures, or bleeding from the gastrointestinal tract can occur. Bleeding is believed to be due to abnormalities of collagen in blood vessel walls leading to vascular fragility. These patients may be mistaken for individuals with hemophilia because of the bleeding tendency and joint abnormalities. Laboratory tests of hemostasis are usually normal in affected individuals. Surgery, trauma, and use of aspirin should be avoided as possible because of bleeding and poor wound healing. Individuals with the vascular type Ehlers–Danlos syndrome are prone to arterial aneurysms and rupture of arterial blood vessels.

Marfan syndrome is an autosomal-dominant genetic disorder due to mutations of the gene for fibrillin resulting in abnormalities of connective tissues and risk for bleeding and

bruising. Individuals with this disorder are usually tall and have long limbs. Dislocation of the lens of the eye, aortic valvular regurgitation, and aortic dissection may occur. Easy bruising or postpartum or postsurgical bleeding may occur but are usually not prominent features of this disorder. Laboratory studies of hemostasis are normal.

Pseudoxanthoma elasticum is an autosomal recessive disorder affecting elastic fibers of connective tissue of the skin and arteries. Several types of the disorder have been described with mutations of a transmembrane transporter protein. The skin of affected individuals has a grooved, thickened appearance. Characteristic hyaline (angioid) streaks are noted in the retina. Easy bruising and gastrointestinal, uterine, and urinary bladder bleeding is common. Bleeding is thought to occur as a result of rupture of calcified blood vessels. Atherosclerosis due to calcification of medium-sized and large blood vessels usually occurs, and intermittent claudication is frequent. Tests of hemostasis are normal, and there is no effective therapy.

Osteogenesis imperfecta is another rare autosomal dominant disorder caused by mutations of genes that code for peptides of type 1 collagen. Skin is mostly type 1 collagen, and defects in supporting structures in skin are believed to be the cause for bruising in this disorder. Four different clinical types of the disorder are reported that are caused by numerous mutations of the genes for these collagen peptides. Affected individuals may demonstrate easy bruising, epistaxis, hemoptysis, and, infrequently, intracranial bleeding. Postoperative wound bleeding can also occur. Reports of benefit with the use of recombinant factor VIIa and desmopressin have been published. Affected individuals have characteristic, blue-colored sclera.

SUMMARY CHART

- Laboratory analysis for quantitative and qualitative platelet disorders includes assays such as platelet count, PFA, PT, aPTT, fibrinogen, and platelet aggregation studies.
- Quantitative and qualitative platelet disorders and vascular disorders are abnormalities of primary hemostasis that result in bleeding and purpura.
- Thrombocytopenia is the most common primary hemostatic disorder.
- The mechanisms responsible for thrombocytopenia include deficient marrow production of platelets, sequestration of platelets in the spleen or hemangiomas, or accelerated loss or destruction of platelets from the circulation.
- Immune thrombocytopenic purpura (ITP) is caused by an autoantibody to a patient's platelets and is often diagnosed based on exclusion.
- Thrombotic thrombocytopenic purpura (TTP) is a microangiopathic process that leads to thrombocytopenia with thrombosis and bleeding.
- Hemolytic uremic syndrome (HUS) presents clinically with microangiopathic hemolytic anemia, thrombocytopenia, and renal failure but unlike TTP levels of ADAMTS-13 are typically normal.
- Qualitative platelet disorders may be caused by abnormalities of the platelet surface glycoproteins (Bernard–Soulier syndrome and Glanzmann's thrombasthenia), deficiencies of platelet granules (storage pool defects), or abnormalities of the platelet release mechanism (release defects).
- von Willebrand disease is one of the most common congenital defects of primary hemostasis.
- von Willebrand factor results in a lack of binding of platelets at the site of injury, with bleeding characteristic of platelet defects.
- Storage pool deficiencies typically present with normal platelet counts and abnormal platelet aggregation tests with all other hemostatic parameters generally normal.
- Platelet morphology and function is typically normal in reactive thrombocytosis where both these parameters are abnormal in primary thrombocytosis.

SUMMARY CHART—cont'd

- Bernard-Soulier syndrome is a deficiency of GPIb/IX/V resulting in impaired platelet activation and adhesion where Glanzmann's thrombasthenia is a deficiency of membrane GP IIb/IIIa complex and results in inability of platelets to bind to fibrinogen.
- Lab results for von Willebrand disease reveal normal platelet morphology and low vWF activity. Patients

with Bernard-Soulier syndrome often have large platelets and platelets that lack aggregation when induced with ristocetin. Hemophilia A is a coagulation factor defect and thus results in normal platelets but abnormal levels of Factor VIII clotting activity.

- Vascular purpura is caused by abnormalities of blood vessels without associated platelet or plasma protein defects.

CASE STUDY 26-1

A 23-year-old African American woman in the 14th week of her first pregnancy presents to the hospital with mild confusion, headaches, nausea, easy bruising, and petechiae. Physical examination reveals a temperature of 37.7°C (99.9° F), blood pressure of 150/100 mm Hg, pulse of 80 beats per minute, and respirations of 14 per minute. Mild confusion and a decrease in light touch sensation of the right arm are noted. Scattered petechiae cover the inner thighs, and ecchymoses on the forearms are present. No other bleeding is observed. The uterus is enlarged, and a fetal heartbeat is detected. The spleen tip is not palpable.

Admission laboratory findings can be seen in the following table. In addition, bone marrow aspiration and biopsy reveal erythroid and megakaryocytic hyperplasia. Hyaline thrombi are seen in blood vessels of the biopsy. Her electrocardiogram and chest radiograph are normal.

Analyte	Findings	Normal Range
Hgb	7.1 g/dL	12.0–16.0 g/dL
Hct	21%	37%–47%
RBC count	$3.6 \times 10^{12}/L$	$4.2\text{--}5.4 \times 10^{12}/L$
WBC count	$10 \times 10^9/L$	$4.8\text{--}10.8 \times 10^9/L$
Plt count	$18.0 \times 10^9/L$	$150\text{--}450 \times 10^9/L$
Peripheral Smear Notes	Moderate poikilocytosis with schistocytes, nucleated red blood cells, and polychromasia	
PT	12.1 secs	11–13 secs
PTT	29 secs	<36 secs
Fibrinogen	367 mg/dL	200–400 mg/dL
FDP	Positive, with a titer of 32	<10 mg/L
D-dimer	Positive at 0.5 to 1.0 mcg/mL	<0.5 mcg/mL
DAT	Negative	Negative
Serum creatinine	2.5 mg/dL	0.84–1.21 mg/dL
Blood urea nitrogen	50 mg/dL	7–20 mg/dL
Calcium	7.6 mg/dL	8.6–10.3 mg/dL
Albumin	2.4 mg/dL	3.4–5.4 mg/dL
Urine protein	3+ positive	Negative
Urine Blood	Positive with RBCs seen microscopically	Negative

QUESTIONS

1. Based on the clinical and laboratory findings, what is the most likely primary hemostatic disorder present?
2. What other conditions should also be considered in the diagnosis?
3. What additional testing could be performed to confirm or reject these diagnoses?
4. How should the patient be treated?

ANSWERS

1. TTP is the most likely diagnosis due to the presence of the classical pentad seen in this disorder: fever, thrombocytopenia, microangiopathic hemolytic anemia, neurological abnormalities, and renal dysfunction. Fever typically presents as less than 38.3°C as seen here. Thrombocytopenia is typically severe, reflected by this patient's very low platelet count. Microangiopathic hemolytic anemia is evidenced by decreases seen in the patient's Hct, Hgb, RBC count, the presence of reticulocytosis, schistocytes, nucleated red blood cells, and polychromasia, and hematuria. In addition, hyaline microthrombi, as seen in this patient's biopsy, is a pathological characteristic of TTP. The patient's mild confusion could be a sign of neurological abnormalities and high levels of creatinine and BUN are representative of renal dysfunction. Patients with TTP typically have normal coagulation tests including PT, PTT, D-dimer, and potentially slight elevations in FDP. TTP is more prevalent in women than men and pregnancy can predispose a woman to the disorder. Cardiac involvement is rare, and this patient has normal ECG findings.
2. Hemolytic-uremic syndrome, pre-eclampsia/eclampsia syndromes, and disseminated intravascular coagulation are other considerations for a pregnant female with microangiopathic hemolytic anemia. Idiopathic thrombocytopenic purpura and gestational thrombocytopenia should also be considered but do not cause microangiopathy as seen in this patient. Additionally, the platelet count in this patient is lower than what is often seen in gestational thrombocytopenia.
3. ADAMTS 13 testing often reveals the presence of autoantibodies to the ADAMTS 13 metalloprotease and

Continued

CASE STUDY 26-1—cont'd

ADAMTS 13 levels are less than 5% in TTP, not in the other disorders.

4. Primary treatment is with plasma exchange. Addition of corticosteroids, cyclosporine A, splenectomy, chemotherapy, or monoclonal antibody immunotherapy (Rituximab) may be necessary for refractory or

chronic relapsing disease. Plasma exchange is the only treatment with proven efficacy and should be instituted as soon as diagnosis is made. Survival rates are 80% to 85%, but relapses occur in 40% of patients. Corticosteroids in addition to plasma exchange may offer additional benefit.

CASE STUDY 26-2

An obese, 36-year-old, white, female emergency medical technician complains of easy bruising of the arms and legs for several months. This bruising is episodic and seems unrelated to trauma during work. She reports no stinging or burning sensations before the appearance of bruises. No bleeding from the nose, mouth, or other mucous membranes is noted. No bleeding into joints or muscles has occurred. Family history is negative for bleeding or bruising. Physical examination reveals normal skin turgor with a few discrete fresh ecchymoses on the thighs and forearms but no other obvious bleeding. Platelet count is $380 \times 10^9/L$; 30 seconds; aPTT, PT, and fibrinogen studies are normal.

QUESTIONS

1. What additional information will be helpful in defining a cause for this bruising?
2. What conditions should be considered in the differential diagnosis? Why?
3. What additional testing can be performed to confirm a diagnosis?

ANSWERS

1. It would be helpful to know if there is any familial history associated with easy bruising to evaluate a potential congenital primary hemostatic disorder. It would

also be helpful to know if the patient is taking any medications, specifically those that can have an effect on hemostasis.

2. Because the platelet count is normal, qualitative platelet disorders should be considered more heavily than quantitative platelet disorders. The patient should be evaluated for conditions like Glanzmann's thrombasthenia, Bernard-Soulier, and von Willebrand's, although the patient's bleeding doesn't match with what is often seen in these conditions. If ruled out, the patient should also be evaluated for vascular conditions, which may include primary purpura, infectious purpura, allergic purpura, or metabolic purpura. Vascular conditions could include primary purpura, infectious purpura, allergic purpura, or metabolic purpura.
3. Thrombin clotting time assay to detect low levels of heparin, von Willebrand factor assays and Factor VIII clotting activity to investigate for mild von Willebrand disease, platelet function studies (PFA assay or platelet aggregation studies) as there could be a platelet function defect. Erythrocyte sedimentation rate, cryoglobulin testing, and serum protein electrophoresis may uncover a vasculitis or hypergamma globulinemic purpura.

CASE STUDY 26-3

A 17-year-old Jewish woman complains of recurrent episodes of bleeding from the nose and mouth since birth and heavy menstrual bleeding since menarche. She has never had bleeding into joints or muscles. Her brother also has a history of nasal bleeding. Physical examination does not reveal any telangiectases of the nose or mouth. Skin and joints appear normal. Gynecological examination is normal. Platelet count is $250 \times 10^9/L$. Appearance of platelets on the peripheral blood smear is normal.

QUESTIONS

1. What conditions should be considered in the differential diagnosis for this bleeding? Why?

2. What additional testing can be performed to evaluate this bleeding tendency?
3. How would each of the possible conditions in the differential diagnosis be treated to minimize further bleeding episodes?

ANSWERS

1. The symptom of mucocutaneous bleeding suggests a platelet function disorder. Coagulation screening test results are not known. von Willebrand disease is the most likely possibility as it is the most common platelet function disorder. Glanzmann's thrombasthenia is uncommon but occurs in individuals of Ashkenazi Jewish

CASE STUDY 26-3—cont'd

- extraction. Bernard-Soulier syndrome patients usually exhibit large platelets.
- Factor VIII clotting activity assay and von Willebrand factor assays to evaluate for von Willebrand disease, platelet function testing to evaluate for other platelet function abnormalities.
 - von Willebrand disease is treated with DDAVP if the disorder is mild, with intermediate purity factor VIII

concentrates which contain functional von Willebrand factor or recombinant von Willebrand factor with factor VIII when severe or when prolonged therapy is necessary. Other platelet function disorders are treated with platelet transfusions or activated factor VII. Antifibrinolytic therapy and estrogen therapy to reduce menstrual bleeding may be helpful.

REVIEW QUESTIONS

- Which of the following laboratory procedures has replaced the Ivy Bleeding Time as the screening test for platelet dysfunction?
 - Flow cytometry
 - PFA-100
 - Platelet antibody testing
 - Lumiaggregation
- Which of the following clinical manifestations is most characteristic of a platelet disorder?
 - Mucosal bleeding
 - Hemarthrosis
 - Retroperitoneal hemorrhage
 - Deep muscle hematomas
- Which of the following is not characteristic of Bernard-Soulier syndrome?
 - Normal to moderately decreased platelet counts
 - Absent platelet aggregation in response to bovine vWF or human vWF plus ristocetin
 - Abnormal platelet aggregation in response to ADP, collagen, and epinephrine
 - Abnormality in platelet membrane GPIb/IX/V
- Which of the following is not characteristic of Glanzmann's thrombasthenia?
 - Deficiency or absence of GPIIb/IIIa
 - Giant platelets with thrombocytopenia
 - Absent platelet aggregation in response to ADP, thrombin, collagen, and epinephrine
 - Normal platelet aggregation to ristocetin and bovine vWF
- Which of the following statements is not true regarding acute immune thrombocytopenia (ITP)?
 - Thrombocytopenia occurs following a viral infection.
 - Spontaneous remissions are common.
 - It is found primarily in young children.
 - Platelet counts are generally higher than in chronic ITP.
- Thrombocytopenia, fever, renal disease, microangiopathic hemolytic anemia, and neurological complications are hallmark characteristics of:
 - Hemolytic uremic syndrome (HUS)
 - Disseminated intravascular coagulation (DIC)
 - Thrombotic thrombocytopenic purpura (TTP)
 - Immune thrombocytopenic purpura (ITP)
- What disorders are classically associated with thrombocytosis?
 - Myeloproliferative neoplasms
 - Autoimmune disorders
 - Immunodeficiency syndrome (HIV)
 - Renal disease
- Which of the following is not an inherited vascular defect?
 - Ehlers-Danlos syndrome
 - Marfan syndrome
 - Amyloidosis
 - Giant hemangiomas
- Which condition is classified as autoimmune thrombocytopenia?
 - DIC
 - ITP
 - Storage pool defects
 - Post-transfusion purpura
- What may be deficient in storage pool diseases?
 - Fibrinogen
 - Von Willebrand factor
 - ADP
 - GP IIb/IIIa

See answers at the back of this book.

Disorders of Secondary Hemostasis

Plasma Clotting Factors

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CHAPTER OUTLINE

Plasma Clotting Factors, Associated Disorders, Laboratory Evaluation, and Treatment

Fibrinogen (Factor I)
Factor II (Prothrombin)
Factor V (Proaccelerin; Labile Factor)
Factor VII (Proconvertin; Stable Factor)
Factor VIII (Antihemophilic Factor) and von Willebrand Factor
Factor IX (Christmas Factor; Plasma Thromboplastin Component [PTC])

Factor X (Stuart–Prower Factor)
Factor XI (Plasma Thromboplastin Antecedent [PTA])
Factor XII (Hageman Factor)
Factor XIII (Fibrin-Stabilizing Factor)
Prekallikrein (Fletcher Factor)
High Molecular Weight Kininogen (Fitzgerald Factor, Williams Factor, Flaujeac Factor)

Circulating Anticoagulants/Acquired Inhibitors

Specific Inhibitors
Nonspecific Inhibitors; The Lupus Anticoagulant and Antiphospholipid Antibodies

Summary Chart

Case Study 27-1

Case Study 27-2

Case Study 27-3

Case Study 27-4

Case Study 27-5

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 27-1 Identify the clotting factor defects that can impair the coagulation system.
- 27-2 Associate the clotting factors with their synonymous names.
- 27-3 Contrast clotting factor deficiencies from platelet-associated disorders.
- 27-4 Categorize laboratory methods as screening tests or differential tests for secondary hemostatic defects.

- 27-5 List the vitamin K–dependent factors.
- 27-6 Compare hemophilia A with von Willebrand disease.
- 27-7 Identify the respective factor deficiency responsible for causing hemophilias A, B, and C.
- 27-8 Assess the common laboratory methods used to identify clotting factor deficiencies.
- 27-9 Contrast specific and nonspecific inhibitors.
- 27-10 Describe laboratory methods used to identify circulating anticoagulants/inhibitors.

This chapter describes the disorders of secondary hemostasis in detail, including the plasma clotting factors, their defects and deficiencies, and how they directly affect hemostasis. Associated disorders and recommended laboratory tests and results are also discussed, as are specific and nonspecific circulating inhibitors and the recommended therapy for each plasma clotting factor defect.

Plasma Clotting Factors, Associated Disorders, Laboratory Evaluation, and Treatment

Clotting factors circulate in the plasma as inactive *zymogens* (precursors of enzymes) known as *serine proteases*, which are proteolytic enzymes that cleave peptide bonds having the amino acid serine in their active sites. The activation of the zymogens within the coagulation pathways leads to the

formation of an insoluble fibrin thrombus, as described in Chapter 25.

Table 27-1 outlines the clotting factors, deficiencies, and corresponding laboratory and clinical findings. Table 27-2 provides the expected laboratory test results related to the clotting factor deficiencies.

Impaired hemostasis due to clotting factor defects can be caused by:

- Decreased production of one or more factors
- Impaired molecular synthesis that interferes with the factor's enzymatic activity (dysfunction)
- Rapid consumption of the coagulation factor(s)
- Interference of one or more factors by circulating inhibitors or antibodies

Bleeding (hemorrhagic) disorders related to clotting factor deficiencies or dysfunction have a clinical presentation that

TABLE 27-1 Clotting Factors, Deficiencies, and Clinical Findings

Factor	Deficiency	Minimum for Hemostasis	Half-life (hours)	Clinical Findings
I	Afibrinogenemia	50–100 mg/dL	120	Umbilical stump bleeding, easy bruising, ecchymosis, gingival oozing, hematuria, poor wound healing
	Hypofibrinogenemia			Mild bleeding episodes
	Dysfibrinogenemia			Possible hemorrhage/thrombosis; possibly asymptomatic
II	Hypoprothrombinemia	30%–40%	100	Postoperative bleeding, epistaxis, menorrhagia easy bruising
V	Parahemophilia	10%	25	Epistaxis, menorrhagia, easy bruising
VII	Hypoproconvertinemia	10%	5	Epistaxis, menorrhagia, cerebral hemorrhage
VIII	Hemophilia A	30%	8–12	May be severe, moderate, mild—spontaneous hemorrhage, hemarthroses crippling, muscle hemorrhage, post-traumatic/postoperative bleeding
	von Willebrand disease and variants: variable inheritance	30%–40%	16–24	Mucosal bleeding, superficial wound bleeding—variable depending on level of F VIII:C, vWF
IX	Hemophilia B (Christmas disease)	30%–40%	20	May be severe, moderate, mild—spontaneous hemorrhage, hemarthroses, crippling, muscle hemorrhage, post-traumatic/postoperative bleeding
X	Stuart-Prower deficiency	10%	65	Menorrhagia, ecchymoses, CNS bleeding, excessive bleeding postpartum
XI	Hemophilia C	20%–30%	65	Mild bleeding, bruising, epistaxis, retinal hemorrhage, menorrhagia
XII	Hageman trait	Unknown	60	Asymptomatic: thrombotic tendency
XIII	Factor XIII deficiency	1%	150	Umbilical cord stump bleeding, poor wound healing, minor injuries causing delayed bleeding, fetal wastage, excessive fibrinolysis, male sterility, intracranial hemorrhage
PK	Prekallikrein (Fletcher factor)	Unknown	35	Asymptomatic
HMWK	Fitzgerald factor	Unknown	156	Asymptomatic

HMWK = high molecular weight kininogen; CNS = central nervous system. *Possible in one-third of factor V deficient patients

Source: From Pittiglio, DH, et al. Treating hemostatic disorders. A problem-oriented approach. In Pittiglio DH: Hemostasis Overview. Arlington, VA: American Association of Blood Banks; 1984, p 28, with permission.

differs from platelet-associated disorders. Most clotting factor deficiencies cause delayed and/or inadequate fibrin formation. Hemorrhage can be more profuse when larger vessels are involved (e.g., during surgery, at birth, or after trauma); the location of the hemorrhage is within muscle, deep-tissue, or joint cavities; or it can be delayed (i.e., a stabilized injury begins oozing or actively bleeding again).

Secondary hemostatic disorders can be inherited as sex-linked or autosomal genetic traits. They can also be acquired due to dietary vitamin K deficiency, liver disease, profuse hemorrhage, or a consumptive coagulopathy (e.g., disseminated intravascular coagulation [DIC]), infection or inflammatory disorders, induction of anticoagulant therapy, or treatment with various drugs.

The laboratory evaluation of secondary hemostatic defects always begins with screening tests: the prothrombin time (PT) and the activated partial thromboplastin time (aPTT). Abnormal results obtained from one or both tests must be followed up with differential tests that should include a fibrinogen level, a thrombin time, and repeating the abnormal PT and/or aPTT on equal mixtures of the patient's plasma with pooled normal plasma, known as **mixing studies**, to further discern the factor(s) causing the abnormality. The mixing studies will normalize, or "correct," the results of an abnormal screening test if there is a deficiency of one or more clotting factors. The inability to immediately correct the screening test result, or a correction followed by prolongation after a 2-hour incubation of the mixed plasmas at 37°C, would suggest the

TABLE 27-2 Laboratory Test Results of Clotting Factor Deficiencies

Factor	BT, PFA-100*	PT	aPTT	TT	Fibrinogen	F XIII Chromogenic Assay
Fibrinogen	ABN	ABN	ABN	ABN	ABN	N/A
II	N	ABN	ABN	N	N	N
V	N/ABN*	ABN	ABN	N	N	N
VII	N	ABN	N	N	N	N
VIII:C	N	N	ABN	N	N	N
vWF	ABN	N	N	N	N	N
IX	N	N	ABN	N	N	N
X	N	ABN	ABN	N	N	N
XI	N	N	ABN	N	N	N
XII	N	N	ABN	N	N	N
XIII	N	N	N	N	N	ABN
Prekallikrein	N	N	ABN*	N	N	N
HMWK	N	N	ABN	N	N	N

*Possible in one-third of factor V deficient patients

**The aPTT will shorten after prolonged contact activation. N = normal; ABN = abnormal; BT, PFA-100* = bleeding time, platelet function assay; PT = prothrombin time; aPTT = activated partial thromboplastin time; TT = thrombin time; vWF = von Willebrand factor; HMWK = high molecular weight kininogen; N/A = not applicable.

Source: Adapted from Pittiglio, DH, et al. Treating hemostatic disorders. A problem-oriented approach. In Pittiglio, DH: Hemostasis Overview. Arlington, VA: American Association of Blood Banks; 1984, p 31, with permission.

presence of an inhibitory substance. The inhibitory substance could be a specific or nonspecific antibody, or an anticoagulant, such as heparin or other direct thrombin inhibitor that has been administered to the patient.

Fibrinogen (Factor I)

Fibrinogen (Factor I) is a large glycoprotein (molecular weight [mw] 340 kD) produced solely by the liver and critical in the final stage of coagulation. It is the most abundant of the plasma coagulation proteins, with a normal plasma concentration of approximately 200 to 400 mg/dL. A minimum of 100 mg/dL is required to maintain normal hemostasis. The normal plasma half-life is approximately 3 to 5 days; up to 5 grams/day are produced by the liver.^{1,2} While the majority of fibrinogen is present in the plasma, it can also be found in interstitial fluid and in platelet alpha granules by absorption from the plasma. The fibrinogen molecule is composed of three pairs of nonidentical polypeptide chains, designated as A-alpha ($A\alpha$), B-beta ($B\beta$), and gamma (γ), joined by disulfide bonds.³ Each chain is encoded by a separate gene on chromosome 4. At the terminal ends of the $A\alpha$ and $B\beta$ chains are two fragments containing negatively charged amino acids known as *fibrinopeptides A and B*. Thrombin, the enzyme created via the activation of prothrombin, cleaves the fibrinopeptides from the fibrinogen molecule producing *fibrin monomers*. This reduces the intermolecular repulsive forces allowing the monomers to aggregate. In the final stage of secondary hemostasis, these soluble fibrin monomers spontaneously polymerize to form soluble, but yet unstable, fibrin strands. These strands self-assemble into an overlapping pattern forming weak lateral associations between them. The production of a stable, insoluble thrombus

requires the formation of covalent cross-linkages between the alpha and gamma chains of the soluble fibrin strands. This is catalyzed by thrombin-activated factor XIII, also known as the *Fibrin-Stabilizing Factor* (see Factor XIII). The stable thrombus is ultimately degraded in vivo by the action of the enzyme *plasmin*, in a process known as *fibrinolysis* (see Chapter 28). In vitro, stabilized fibrin is resistant to enzymatic digestion or chemical degradation.

Disorders of fibrinogen are inherited or acquired, and they can involve quantitative and/or qualitative defects.

Afibrinogenemia

Afibrinogenemia is a rare (estimated prevalence 1:1 million) homozygous, autosomal recessive disorder. It is characterized by the absence of any chemically, antigenically, or functionally detectable fibrinogen in the plasma. Fibrinogen levels may be less than 5 mg/dL. There is profuse bleeding after slight trauma and delayed wound healing. The severity of the disease presents at birth with symptoms that include bleeding from the umbilical cord stump, mucosal tissues (e.g., epistaxis, gastrointestinal bleeding), as well as intracranial hemorrhages, which can be fatal. Other symptoms can include menorrhagia and hemarthroses. Platelet adhesion and aggregation are abnormal: fibrinogen is required for normal primary platelet aggregation, as it binds the glycoprotein IIb/IIIa (GP IIb/IIIa) receptor on stimulated platelets, forming a linkage with adjacent platelets. This reinforces the platelet plug at the site of vascular injury (see Chapter 25).¹

The differential diagnoses of fibrinogen disorders are outlined in Table 27-3. The results of these tests will show marked abnormalities: terminal prolongation (no detectable

TABLE 27-3 Differential Diagnoses of Fibrinogen Disorders

Test	Afibrinogenemia	Hypofibrinogenemia	Dysfibrinogenemia
BT PFA-100*	ABN	N	N
PT	ABN	ABN	N/ABN
aPTT	ABN	ABN	N/ABN
Thrombin time	ABN	ABN	ABN
Reptilase time	ABN	ABN	ABN
Fibrinogen (clottable)	Undetectable	ABN	N/ABN
Fibrinogen (antigen)	Absent	ABN	N
Platelet aggregation	ABN	N	N

N = normal; BT, PFA-100* = bleeding time, platelet function assay; ABN = abnormal; aPTT = activated partial thromboplastin time; PT = prothrombin time. N/A = not applicable.
Source: From Girolami, A, et al. Rare and quantitative and qualitative: Abnormalities of coagulation. *Changes Hematol*. 1985;14:388, with permission.

endpoint) of the PT, aPTT, and TT, and the absence of measurable fibrinogen. Mixing studies will demonstrate a normalization of these results.

Treatment for afibrinogenemia requires the administration of cryoprecipitate; this preparation contains appreciable amounts of fibrinogen. Fresh frozen plasma (FFP) may be used; however, volume overload is a major concern due to the number of units required to sufficiently elevate the fibrinogen level. Whole blood transfusions may be required if significant bleeding has occurred.

Hypofibrinogenemia

Hypofibrinogenemia can occur as either autosomal recessive or heterozygous dominant forms of disorders; mutations may exist in one or more of the genes that direct the rate of synthesis of the 3 polypeptide chains. The fibrinogen level may be within 20 to 100 mg/dL. Any bleeding episodes are usually mild and do not occur spontaneously. Bleeding may occur after trauma or surgery.

Hypofibrinogenemia can be acquired as a result of liver disease, due to consumption in cases of acute DIC, or from hemodilution as a result of massive transfusion.

The degree to which the PT, aPTT, and TT are prolonged is dependent on the concentration of fibrinogen present in the plasma.

Treatment of hypofibrinogenemia may include the use of cryoprecipitate and/or FFP as necessary.

Dysfibrinogenemia

A qualitative functional disorder of fibrinogen, dysfibrinogenemia involves an alteration in the structure of the molecule, either in its amino acid sequence or carbohydrate composition, leading to the formation of an abnormal glycoprotein. The alterations are caused by mutations in the genes that direct the synthesis of the 3 polypeptide chains. These changes can affect the normal interactions of fibrinogen with its enzymes and cofactors, thereby causing defective thrombus formation as well as impaired fibrinolysis.

Dysfibrinogenemia can be inherited as autosomal dominant or recessive traits. More than 300 different structural variants have been identified. These molecules may exhibit abnormal

fibrinopeptide release by thrombin, abnormal polymerization of the monomers, defective cross-linking, or a combination of these defects.

Acquired dysfibrinogenemia can occur in chronic liver disease, chronic malignancies, or autoimmune diseases. These disorders may induce relatively minor structural changes in the molecule, producing a functional dysfibrinogenemia with similarities to the congenital forms.

Patients with dysfibrinogenemia can have a normal or prolonged PT and aPTT depending upon the severity of the dysfunction. Bleeding time and platelet function are unaffected. Specific testing of fibrinogen will usually be abnormal. Polymerization of fibrin monomers induced by treating the abnormal molecules with a high concentration of thrombin (i.e., the Clauss clot-based fibrinogen assay; see Chapter 33), has been found to be normal in several cases.⁴ However, with the lower thrombin concentrations present in the TT test (increasing the sensitivity to defects in the conversion of fibrinogen to fibrin), polymerization is delayed due to the slower cleavage rate of the abnormal peptide, producing an abnormal result.⁵ A common laboratory finding is a discordance in the activity and antigenic concentration: decreased to normal level of functional fibrinogen with a higher concentration of the antigenic protein.

Anticoagulants such as heparin, hirudin (a direct thrombin inhibitor derived from leeches), and synthetic direct thrombin inhibitors will prolong the TT. To evaluate the cause of an abnormal TT, a Reptilase Time (RT) should be determined.⁶ **Reptilase**, a thrombin-like enzyme extracted from the snake venom of *Bothrops atrox* (Pit Viper species), only cleaves fibrinopeptide A from the fibrinogen molecule and forms fibrin monomer. It can be used to differentiate between the presence of heparin, antithrombin anticoagulants, or dysfibrinogenemia.⁷ Reptilase is unaffected by heparin, hirudin, and antithrombins; therefore, the clotting time will be normal in their presence, provided an adequate amount of normal fibrinogen is present (see Table 27-3). Hypofibrinogenemia and afibrinogenemia will also have prolonged RTs.

Clinically, patients with dysfibrinogenemia are usually asymptomatic; the condition is often discovered incidentally

when laboratory tests are performed for unrelated reasons. Occasionally, it is associated with mild bleeding occurring only after trauma. However, there are reports indicating an association with thrombosis.⁸ This may be a result of abnormalities in the interaction of plasmin with the abnormal fibrin thrombus, causing a resistance to lysis.

Treatment for dysfibrinogenemia may involve FFP or cryoprecipitate if bleeding occurs. If recurrent thrombosis exists, anticoagulant therapy is indicated.⁹

Hyperfibrinogenemia

Fibrinogen is also an acute-phase reactant. Physiological stresses such as trauma, pregnancy, and tissue inflammation cause fibrinogen concentrations to increase, which is manifested in an elevated erythrocyte sedimentation rate. High fibrinogen concentrations may be seen in acute hepatitis and may also be associated with atherosclerosis, arterial thrombosis, or both.

CRITICAL THINKING QUESTION

- 27-1** Why does a decrease in fibrinogen, like that seen in afibrinogenemia or hypofibrinogenemia, result in prolonged PT and PTT findings, yet the mixing study is corrected?

See answers to all Critical Thinking Questions at the back of this book.

Factor II (Prothrombin)

Factor II (F II), synonymously known as **prothrombin** (mw 71.6 kD), is a single-chain glycoprotein synthesized in the liver. It is the most abundant clotting factor, has the longest half-life of the vitamin K-dependent clotting proteins, and circulates as a zymogen to the serine protease thrombin (Factor IIa).¹⁰ F II is converted to thrombin (F IIa) by the proteolytic action of activated factor X (F Xa) in complex with activated factor V (F Va), platelet membrane phospholipids, and calcium ions. This is known as the **Prothrombinase Complex**, and it assembles on the platelet surface. The generally accepted normal adult reference range is 50% to 150% (0.5 to 1.5 U/mL), although it may vary by institution or published reference. Deficiency of F II delays the generation of thrombin, thus contributing to hemorrhagic symptoms. The Prothrombin Time (PT) is an in vitro analysis of the time required to convert plasma prothrombin into endogenous thrombin, using an exogenous source of phospholipid, tissue thromboplastin (also known as tissue factor), and calcium ions. The fibrinogen present in the plasma is converted to a fibrin endpoint. This assay detects defects or deficiencies of any components of the prothrombinase complex, and by the concentrations of fibrinogen, F II, and factor VII (F VII).

Hypoprothrombinemia

It has been suggested that the mode of inheritance of hypoprothrombinemia is autosomal recessive.¹¹ The gene resides on chromosome 11. Patients who are either heterozygous or homozygous with this rare condition may have hemorrhagic symptoms with prothrombin levels from 2% to 25% of normal

activity. Deficiency may also be acquired by dietary vitamin K deficiency or oral anticoagulant (warfarin) therapy. However, the type and severity of symptoms may vary with the level of functional prothrombin available. Epistaxis, menorrhagia, postpartum hemorrhage, hemorrhage after surgery or trauma, and broad-spectrum antibiotic use (which destroy the normal flora of the gastrointestinal tract responsible for vitamin K synthesis) are exhibited with prothrombin levels ranging from less than 2% to less than 50%. Prothrombin levels approaching 50% activity generally do not cause hemorrhagic symptoms. Variable prolongation of both the PT and aPTT, and a normal TT, are observed in individuals with hypoprothrombinemia. These screening assays are not specific for hypoprothrombinemia. Definitive diagnosis is dependent on specific assays for functional activity and/or antigenic concentration of prothrombin. In hypoprothrombinemia, both the functional activity and antigenic concentration of prothrombin are decreased. Mixing studies will show full correction of the PT and aPTT (see Table 27-2).

Treatment of hypoprothrombinemia may include the administration of FFP or *prothrombin complex concentrate* (PCC), which contains factors II, VII, IX, and X. Vitamin K supplementation will only restore low factor levels associated with dietary deficiency or oral anticoagulant therapy.

Dysprothrombinemia

The mode of inheritance in dysprothrombinemia is the same as in hypoprothrombinemia.¹² A structural defect causes impaired functional activity, although the antigenic concentration is normal. Bleeding manifestations may occur that are similar to those described for hypoprothrombinemia. Vitamin K deficiency, induction and therapeutic warfarin therapy, liver disease, or the presence of antibodies to prothrombin must be differentiated from dysprothrombinemia and hypoprothrombinemia to determine the proper course of therapy. Treatment may include administration of FFP or PCC.

Prothrombin G20210A Mutation

A single-point mutation in the prothrombin gene was discovered in 1996 that was associated with a mildly elevated prothrombin concentration and an increased risk of venous thrombosis.¹³

ADVANCED CONTENT

The mutation, known as *Prothrombin G20210A*, consists of a single base change resulting from a G (guanine) to A (adenine) transition at nucleotide 20210, in the 3'-untranslated region of the prothrombin gene.¹⁴

It is the second most common cause of inherited thrombophilia (i.e., predisposition to thrombosis) (see also *Factor V Leiden mutation* later). Patients who are heterozygous for this mutation carry a two- to five-fold increased risk of venous thromboembolism compared with normal individuals. This genetic defect is not commonly found in patients with

arterial thromboembolic disease, but it has been identified as a risk factor for myocardial infarction in young women and cerebrovascular ischemic disease in young patients.^{15,16} This autosomal dominant gene is predominantly (although not exclusively) restricted to the Caucasian population, occurring in approximately 1% to 2%. In the presence of other risk factors such as pregnancy, oral contraceptives, acquired risk factors such as antiphospholipid antibody (lupus anticoagulant), recent surgery, or trauma, the incidence of thrombosis is increased.^{17,18}

The elevation of F II activity, which may only be slightly greater than levels in patients without the mutation (approximately 115% to 130%), may still be within normal limits, making this an insensitive method of detection. Therefore, the mutation can only be detected by direct analysis of genomic DNA. Polymerase chain reaction (PCR) technology is only one (initial) part of analyzing such single nucleotide polymorphisms.

Factor V (Proaccelerin; Labile Factor)

Factor V (F V) is a single-chain glycoprotein (mw 350 kD) synthesized in the liver and also present in the alpha granules of platelets that are secreted during platelet activation.^{19,20} It has a short half-life in vivo and has been referred to as the "labile factor" because of its rapid deterioration of activity in plasma at room temperature. Historically, F V has also been known as *proaccelerin*, as it is the catalyst in the conversion of F II to F IIa, functioning as a cofactor within the prothrombinase complex. It is converted to its activated form, F Va, by the actions of F IIa or F Xa with calcium ions and platelet phospholipids. The generally accepted normal adult reference range is 50% to 150% and may vary by institution or published reference.

In 1947, Owren discovered F V deficiency in a 21-year-old woman with a lifelong bleeding history.^{21,22} Studies demonstrated that this clotting factor was not vitamin K-dependent. Congenital F V deficiency, also known as *parahemophilia*, is inherited as an autosomal recessive trait.²³ The gene for F V is carried on chromosome 1. It is an extremely rare condition that occurs with a probability of 1:1 million.²⁴

Deficiency of F V is associated with ecchymoses, epistaxis, menorrhagia, and gingival, gastrointestinal, umbilical, and central nervous system bleeding. Hemorrhagic manifestations are usually noted in individuals with less than 10% activity. Hemarthrosis seldom occurs even in severely deficient patients. Combined deficiencies of factors V and VIII have been reported in several families. The combined factor V and VIII activities and antigen levels are decreased, ranging from 5% to 30% of normal.²⁵ This syndrome has been found to be the result of a gene mutation that produces a protein that affects the intracellular transport of these factors from the endoplasmic reticulum to the Golgi apparatus. Its defective transport leads to reduced secretion of both coagulation factors.²⁴

Acquired F V deficiency is associated with a variety of disorders such as liver disease, carcinoma, tuberculosis, and DIC.²⁶ It can also result from the presence of F V-specific antibodies acquired after childbirth, due to autoimmune diseases,

or by exposure to certain types of topical bovine thrombin ("fibrin glue") used for intraoperative hemostasis during surgical procedures. Bovine F V has been found to be present in the commercial product and is suggested that the antibody to the bovine F V cross-reacts with human F V.²⁷

Laboratory evaluation demonstrates a prolonged PT and aPTT, with correction for both tests on mixing studies. The TT is normal. Approximately one-third of patients may present with an abnormal platelet function test (PFA-100), possibly related to platelet alpha granule-associated F V deficiency.²⁸ Plasma factor activity by clotting factor assay is decreased or absent. Noncorrection of a mixing study will occur in the presence of an inhibitor.

F V-deficient patients are often treated with FFP. In addition to FFP, because platelets contain F V, platelet transfusions have also been used; however, particular attention should be paid to alloimmunization.²⁹ Cryoprecipitate does not contain adequate amounts of F V to be used therapeutically.

Factor V Leiden Mutation (R506Q)

Under normal hemostatic conditions, F Va (and, as will be discussed, F VIIIa) must be inactivated in vivo to downregulate further thrombin generation. This is achieved by the naturally occurring anticoagulant, *Protein C*, and its cofactor, *Protein S*. Protein C becomes activated by thrombin bound to its receptor on the endothelial cell surface known as the *Thrombomodulin* complex (see Chapter 29). Activated Protein C (APC) is responsible for the inactivation of F Va and F VIIIa.

In 1993, a new and very common inherited thrombophilic syndrome was described, *Activated Protein C Resistance (APCR)*. A single point mutation was identified involving a guanine (G) to adenine (A) transition at nucleotide 1691 of the F V gene, resulting in the substitution of arginine (R) by glutamine (Q) at amino acid number 506. This is one of the cleavage sites where APC must bind to the factor V molecule.³⁰ This mutation was named *Factor V Leiden (R506Q)* by Dutch investigators from the city of Leiden, Holland, who were the first to report this mutation.³¹ This substitution obliterates the binding site leading to impaired inactivation (resistance) of F Va by APC, resulting in continued thrombin generation and promoting thrombosis (Fig. 27-1). Heterozygous Factor V Leiden is present in Caucasians ranging between 1.0% to 8.5%, depending on geographic population studied, but it is at least 10 times more prevalent than other known genetic thrombophilic disorders.^{32,33} These patients are unlikely to develop thromboses unless there are additional risk factors present that have an additive effect (e.g., oral contraceptives, estrogen replacement therapy, pregnancy, nonambulatory state, or trauma). It has not been detected in populations from Africa, Asia, and Australia or in Native Americans.³⁴ This mutation is responsible for more than 90% of the cases testing positive for APCR; approximately 40% to 50% of patients with recurrent venous thrombosis were found to have the mutation.³¹ Homozygosity for the mutation alone, as well as heterozygosity combined with other related thrombophilic causes, will cause a much higher thrombotic risk. The clottable activity of F V is unaffected by this mutation. Detection of the R506Q defect can only be accomplished by genomic DNA analysis.

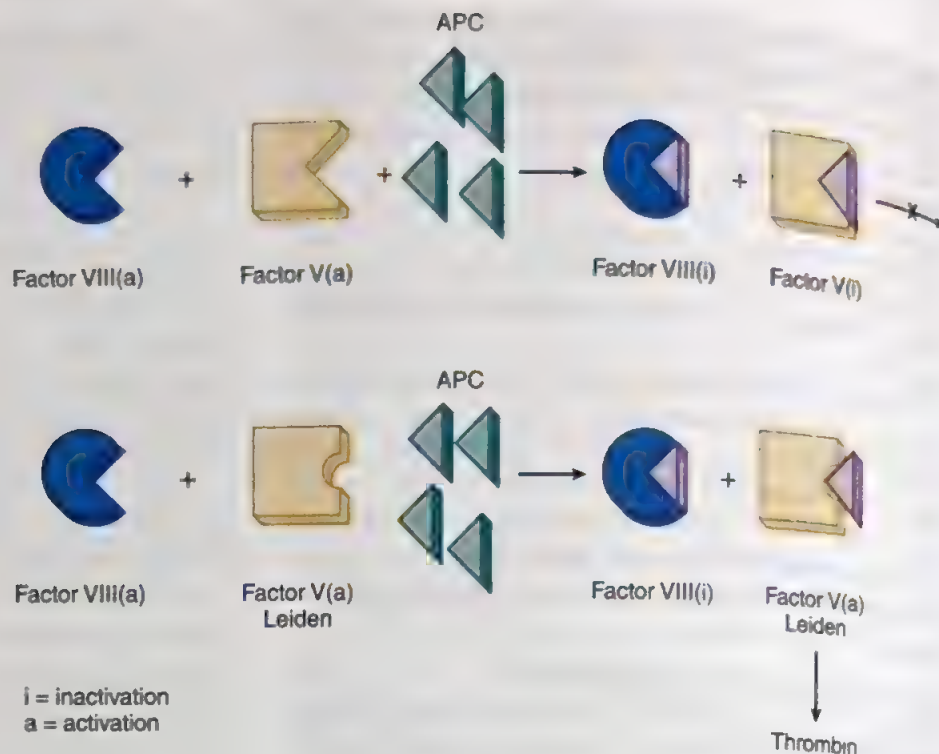


FIGURE 27-1 Activated protein C (APC) resistance of factor V Leiden. (Top) Normal inactivation of factors V and VIII:C by APC. (Bottom) The binding site for APC on the factor V Leiden molecule is altered, thereby permitting activated factor V (factor Va Leiden) to continue thrombin generation and subsequent fibrin formation.

ADVANCED CONTENT

APCR has been found in a small percentage of patients (approximately 10%) without the factor V Leiden (R506Q) mutation.³⁵ A mutation at Arg 306 (an additional APC cleavage site in F Va) has been characterized.^{36,37} There are also cases of acquired APCR associated with malignancies, lupus anticoagulants, and third trimester pregnancy. This illustrates the importance of detecting APCR in patients who may be at an increased risk of thromboembolic disease.

Screening for APCR is easily performed by clottable assays utilizing modified aPTT or Russell's viper venom time methods. By either method, the plasma clotting time with the addition of APC is compared with the clotting time of the plasma without added APC. Normal patients produce prolonged clotting times in the presence of APC, demonstrating its ability to inactivate F Va and F VIIIa; the clotting time without APC will therefore be significantly shortened. Patients with APCR will produce shortened clotting times for both tests, indicating the inability of APC to exert its effect on F Va or F VIIIa, hence the description "resistance to APC" (refer to Chapter 28 for a further discussion of the technique).

Factor VII (Proconvertin; Stable Factor)

A predominant plasma protein of the extrinsic coagulation pathway is F VII (mw 50 kD), a heat- and storage-stable component for which it is also known as the "stable factor." It is also known as *proconvertin* for its pivotal role in coagulation. It is produced by the liver and is vitamin K-dependent; it circulates as an inactive zymogen, as well as in low levels of its activated form, F VIIa.³⁸ The normal adult reference range is generally accepted as 50% to 150%.

The division of the coagulation cascade into the intrinsic and extrinsic pathways was created as a useful tool for laboratory diagnoses; however, it does not exist as such in vivo. Initiation of coagulation in vivo involves components of the vasculature and platelets. A major component is **tissue factor (TF)**, also known as tissue thromboplastin, functioning as a cofactor. Tissue factor is a transmembrane lipoprotein normally found exclusively in the extravascular space; it is also synthesized by monocyte/macrophages and endothelial cells. Its surface expression can be induced by inflammatory cytokines; this seems to be one of the most important pathophysiological mechanisms of DIC in sepsis. It is also present in many other tissues, such as brain, lung, and placenta. In the event of vessel injury, TF and F VII/F VIIa form a complex (TF/F VIIa) in the presence of calcium ions. This complex, known as an *extrinsic tenase complex*, activates F X to F Xa. The TF/F VIIa complex is also capable of activating F IX, bypassing the need for contact activation of factors XI and XII. Positive feedback mechanisms exist that further activate more F VII.

Homozygous F VII deficiency is a rare, autosomal-recessive trait (1:500,000 estimated incidence),^{39,40} and is associated with missense mutations of the F VII gene on chromosome 13.⁴¹ F VII activity is usually less than 10%. Clinically, these patients can present with deep muscle hematomas, hemarthroses, epistaxis, and menorrhagia. Patients having less than 1% F VII activity can have severe hemorrhagic manifestations. Heterozygotes are usually asymptomatic, having factor activities between 40% and 60%.

Typical laboratory results in F VII deficiency include a prolonged PT with a normal aPTT (Table 27-4). The PT will correct on mixing study. No longer in use for differential testing but worth noting (more recent usage for inhibitor testing will be discussed; see Non-Specific Inhibitors—Lupus Anticoagulants), testing with a Russell's viper venom reagent known as the Stypven time will produce a normal clotting

TABLE 27-4 Laboratory Testing and Results for Factor VII Deficiency

Test	Results	Reference Range
PT	>14 sec	10.0–14.0 sec*
aPTT	<36 sec	23–36 sec*
Factor VII assay	<50%	50–150%
Stypven time	<25 sec	<25 sec*
Other factor activities	>50%	50–150%

PT = prothrombin time; aPTT = activated partial thromboplastin time; sec = seconds.
 *Generic reference range for illustrative purpose

time in F VII deficiency because the venom directly activates F X, bypassing the extrinsic pathway completely. Documentation of F VII deficiency requires the performance of a F VII activity assay.

Factor VII deficiency can be acquired with liver disease, warfarin therapy, broad-spectrum antibiotic use, or dietary vitamin K deficiency. Treatment can include FFP, PCC, and/or vitamin K supplementation. In addition, a genetically engineered preparation structurally similar to human plasma-derived F VIIa, recombinant activated factor VII (rFVIIa) can be used in congenitally deficient patients with a hemorrhagic diathesis. rFVIIa was approved by the FDA in 1999 as a treatment option in hemophiliacs with inhibitors (see Hemophilia A; see also Circulating Anticoagulants/Acquired Inhibitors).

Factor VIII (Antihemophilic Factor) and von Willebrand Factor

Factor VIII (F VIII) is a large glycoprotein (mw 330 kD), essential for the normal rate of thrombin production and critical to maintaining hemostasis. The liver is the site of F VIII synthesis but not by hepatocytes; severe hepatic failure does not cause F VIII deficiency.^{42,43} Some evidence

suggests that hepatic sinusoidal endothelial cells are the major site of synthesis.⁴⁴

F VIII (also known as the *antihemophilic factor*) is secreted into the plasma, where it circulates as a macromolecular complex with von Willebrand factor (vWF), designated as F VIII:vWF.⁴⁵ vWF is synthesized within megakaryocytes and endothelial cells as a high molecular weight (HMW) monomeric glycoprotein (mw 250 kD) that dimerizes and polymerizes into even higher molecular weight molecules known as *multimers*. vWF is stored and secreted from granules within endothelial cells, known as *Weibel-Palade bodies*, and from the alpha granules in platelets.⁴⁶ These multimers are secreted and circulate in the plasma as a heterogeneous mixture, with molecular weights ranging from 600 to 20,000 kD.⁴⁷ The plasma half-life is approximately 24 hours. Only 1% to 2% of the F VIII:vWF complex functions as the clotting factor, or *coagulant*, designated as F VIIIc. This fraction is measurable by specific factor assay. F VIIIc has a short half-life of approximately 8 to 12 hours while bound to vWF. It functions as a cofactor, having no enzymatic activity of its own. Proteolytic cleavage, by tissue factor-initiated thrombin generation, activates F VIIIc to F VIIIca. It then dissociates from vWF, allowing it to function as the cofactor to activated F IX (F IXa) and react with platelet phospholipids and calcium ions to form the *intrinsic tenase complex*; this accelerates the conversion of F X to F Xa (see Chapter 25). The remaining portion of the F VIII:vWF complex mediates platelet adhesion and also stabilizes F VIIIc, protecting it from enzymatic degradation or inhibition. The F VIII:vWF complex serves as a carrier to concentrate F VIIIc at the site of vessel damage. After vessel injury, vWF interacts with exposed subendothelial collagen and the glycoprotein Ib/IX/V (GP Ib/IX/V) receptors on the platelet membrane, resulting in platelet adhesion to the vascular subendothelium. This reaction, coupled with the fibrin-forming action of F VIIIc at the same site, is the critical process of hemostasis, (i.e., the formation of the hemostatic plug). Selected properties of F VIIIc and vWF are summarized in Table 27-5.

TABLE 27-5 Selected Properties of Factor VIIIc and von Willebrand Factor*

Property	Factor VIIIc	von Willebrand Factor
Site of biosynthesis	Hepatic sinusoidal epithelial cells	Endothelial cells, Megakaryocytes
Plasma concentration	50%–150%	50%–150%
Molecular weight	330 kD	600–20,000 kD
Principal biological activity	Procoagulant; cofactor; Intrinsic tenase assembly	Platelet adhesion to vessel wall
Functional assay	aPTT; factor VIII Assay	BT, PFA-100*, RCA, automated latex agglutination, RIPA
Antigenic assay	IRMA, ELISA	ELISA; Immunoelectrophoresis; automated latex agglutination
Inheritance	Sex-linked recessive	Autosomal
Clinical disorder caused by deficiency	Hemophilia A	von Willebrand disease

*In plasma, the two proteins are present as a macromolecular complex: F VIII:vWF complex

BT, PFA-100* = bleeding time, platelet function assay; RCA = ristocetin cofactor assay; RIPA = ristocetin-induced platelet agglutination; IRMA = immunoradiometric assay, ELISA = enzyme-linked immunosorbent assay.

Source: From Marder VJ, et al. Standard nomenclature for factor VIII and von Willebrand factor: A recommendation by the International Committee on Thrombosis and Hemostasis. *Thromb Haemost*. 1985;54:871, with permission

The generally accepted normal adult reference range for both F VIIIIC and vWF is 50% to 150%, with 40% considered the minimum to maintain hemostasis. Defects and/or deficiencies of F VIIIIC and vWF cause hemorrhagic disorders with variable severities, known as *hemophilia A* and *von Willebrand disease (vWD)*, respectively. Each disorder is discussed separately in detail.

F VIIIIC and vWF are also acute-phase reactants; levels increase during the inflammatory response to infection, surgery, cancer, trauma, or pregnancy. Inactivation of F VIIIa by APC is required to prevent ongoing thrombin generation and thromboembolic complications. During some inflammatory processes, an elevated factor level in combination with other vascular and activated platelet-derived substances may promote venous thrombosis.

Oral contraceptives and estrogen replacement therapy can also elevate F VIIIIC and vWF levels. If there are predisposing factors such as surgery, trauma, systemic infection, immobilization, or an unrecognized abnormality of the anticoagulant-fibrinolytic mechanism, the risk of pulmonary embolism, deep vein thrombosis, or stroke increases dramatically.

The laboratory assessment of F VIIIIC and vWF should include assays of specific factor activity as well as vWF functional activity, antigenic concentration, and multimeric structure. In addition, platelet function assays may be included in some cases to effectively evaluate the platelet-vWF interactions. Each of the assay types are addressed in the sections that follow.

F VIIIIC

F VIIIIC activity is expressed in the global test of the intrinsic pathway, the aPTT. Screening studies by performing the aPTT will be prolonged if the level is decreased or absent. A mixing study will normalize the aPTT. Quantitative analysis requires an aPTT-based or chromogenic specific factor activity assay. Specimen stability is limited; F VIIIIC is thermolabile and rapidly loses activity at room temperature.

Von Willebrand Factor

The function of vWF cannot be determined by standard clotting assays because it is not involved in fibrin formation. A method that qualitatively assesses the platelet-vWF functional interaction is known as the *ristocetin-induced platelet agglutination (RIPA) assay*. This method utilizes an *aggregometer*, a device that records the change in optical density of a platelet suspension as agglutination occurs in response to stimulating agents (agonists). RIPA assays evaluate vWF binding with GP Ib/IX/V.

ADVANCED CONTENT

The vWF-platelet GP Ib/IX/V response is simulated in vitro using platelet-rich plasma and a substance known as *ristocetin*, a glycopeptide antibiotic (structurally similar to vancomycin) first used clinically in the late 1950s. Very soon after its release, ristocetin was found to cause thrombocytopenia; therefore, clinical use was discontinued. It was not

until 10 years later that it was discovered to agglutinate normal platelets in vitro but cause little to no agglutination in some patients known to have vWD.⁴⁸ Studies suggest that ristocetin reduces the negative charge at the platelet membrane surface, reducing the repulsive forces between platelets and/or between platelets and vWF, promoting platelet agglutination.⁵⁰ It also induces a conformational change in vWF to promote binding to GP Ib/IX/V. Normal hemophilic plasma added to vWF-deficient plasma would induce agglutination due to the presence of vWF. The RIPA assay uses ristocetin at final concentrations between 1.2 to 1.5 mg/mL. Altering the concentration of ristocetin (e.g., 0.2 to 0.6 mg/mL), known as the *low-dose RIPA method (LD-RIPA)*, is useful in detecting variant forms of vWF. RIPA is advantageous in detecting both vWD and disorders related to membrane GP Ib/IX/V defects (i.e., Bernard-Soulier syndrome; see Chapter 26). A snake venom from the *Bothrops* species, known as *botrocetin*, can also produce vWF-dependent agglutination, but its action differs from ristocetin in that it first binds to vWF, then the complex vWF/botrocetin binds to the GP Ib/IX/V receptor, causing agglutination. Ristocetin only reacts with the high molecular weight (HMW) multimer fraction, whereas botrocetin is useful to determine the reactivity of vWF with GP Ib/IX/V regardless of the multimeric structure.⁴⁹

The RIPA assay is not sufficiently sensitive to mild reductions of vWF; therefore, it only serves as a qualitative indicator. A method that quantitatively measures plasma vWF activity, known as the *ristocetin cofactor activity assay (RCA or vWF-R:CoF)*, also utilizes an aggregometer to determine the rate of agglutination of a standardized suspension of formalin-fixed normal platelets, ristocetin (1.0 mg/mL), and patient plasma. The rate of agglutination of this mixture is compared with a calibration curve, constructed by plotting the slopes of the reaction tracings on dilutions of an assay reference plasma versus the percentage of vWF activity. However, this method is time-consuming, labor-intensive, and imprecise (i.e., a coefficient of variation [CV] as high as 15%).⁵¹ Currently, the RCA method can be adapted on some automated analyzers. Latex immunoturbidimetry is also available, utilizing latex-bound GP Ib/IX/V binding to vWF. These methods provide a more sensitive, precise, rapid, and automated measurement of vWF-dependent function.

In the RCA method, the formalin-fixed platelets are not metabolically active; therefore, the response to ristocetin is *agglutination*. In the RIPA method, the patient's metabolically active (viable) platelets are exposed to ristocetin. Agglutination initially occurs, but subsequent physiologic changes result in the release of endogenous ADP and other platelet-derived agonists. This response is considered *aggregation*; once vWF binds to GP Ib/IX/V, it induces the subsequent GP IIb/IIIa receptor-dependent aggregation response (Fig. 27-2). GP IIb/IIIa is normally the physiological binding site for fibrinogen during in vivo aggregation, but in the

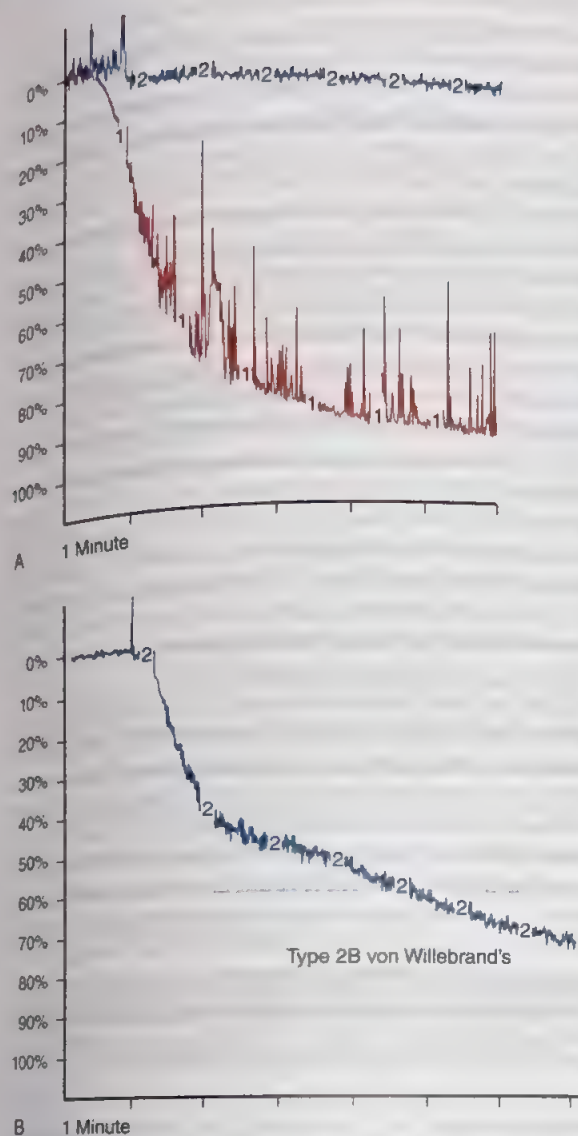


FIGURE 27-2 Ristocetin-induced platelet aggregation. A. (1) Normal response to ristocetin 1.2 U/mL. (2) Normal response to ristocetin 0.6 U/mL (low-dose). B. Abnormal response to ristocetin 0.6 U/mL characteristic of type 2B von Willebrand's disease. Each mark on the x axis represents 1-minute intervals.

absence of fibrinogen or when vWF is at high concentrations, such as at the site of vascular injury, vWF may also induce this receptor-mediated aggregation process.⁴³ The RIPA assay will produce an aggregation and secretion response in normal patients.

The concentration of plasma vWF antigen can be determined by several methods: ELISA; Laurell rocket immunoelectrophoresis (IEP), using an anti-vWF:Ag-impregnated agarose gel (Fig. 27-3); and rapid automated methods, utilizing anti-vWF-coated latex particles.

vWF multimer analysis of plasma or platelet lysate is performed using sodium dodecyl sulfate (SDS)-agarose-gel electrophoresis, followed by Western blotting with enzyme-linked antibodies, and then staining to visualize the banding pattern

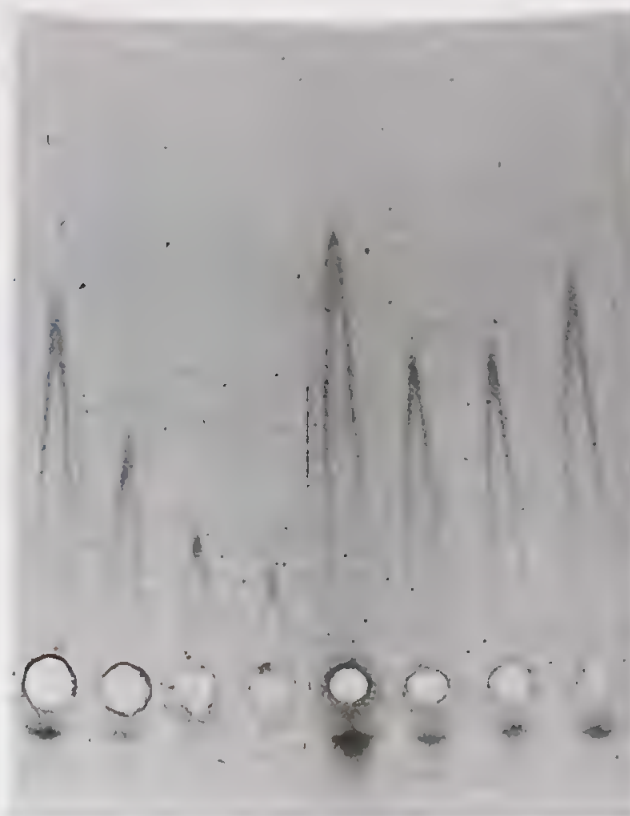


FIGURE 27-3 Quantitative immunoelectrophoresis of vWF:Ag; Laurell rockets in agarose gel. The first four peaks are the standard curve dilutions, followed by various vWF:Ag levels. Peak height is proportional to concentration.

(Fig. 27-4). The multimers are separated on the basis of molecular weight. A normal distribution pattern shows a progression from the smallest to the largest multimers.⁵² Defects in the banding pattern are visually apparent. Quantitative deficiencies of vWF demonstrate decreased staining intensity or complete absence of banding; in qualitative defects, the absence of high and/or intermediate molecular weight multimer bands is noticeable.

Hemophilia A

Hemophilia A (classic hemophilia) is a hereditary coagulopathy, second in overall frequency of the inherited bleeding disorders after von Willebrand disease. It is, however, the most common of the hemophilic syndromes (hemophilias A, B, and C). Hemophilia A is a sex-linked recessive bleeding disorder that has been documented for centuries. Approximately 1 in 5,000 to 10,000 affected males are born annually. The fifth-century Talmud described a bleeding episode that occurred after circumcision. Modern rabbinic command forbids the circumcision of any child in whom the diagnosis of hemophilia has been made.⁵³ The disorder was found in the royal families of Europe—Great Britain, Spain, and Russia—via Queen Victoria who was a carrier and the source of hemophilia in four subsequent generations.^{50,54}

Hemophilia A is caused by a molecular defect or absence of the coagulant portion of the F VIII complex, F VIIIc, due



FIGURE 27-4 vWF multimers. Samples of (1) normal plasma, (2) von Willebrand's plasma, and (3) cryoprecipitate, underwent electrophoresis in sodium dodecyl sulfate (SDS)-agarose gel. A Western blotting technique was performed. The gel on nitrocellulose paper was incubated first with a rabbit antihuman vWF:Ag antibody and then with goat antirabbit IgG, after which it was stained.

to (1) a point mutation involving a single nucleotide; (2) deletion of all or part of the gene; or (3) a mutation affecting gene regulation.⁵⁵ The vWF component (vWF:Ag) of the F VIII complex is found to be normal in patients with hemophilia A. The gene for F VIIIIC resides on the X chromosome; therefore, males inheriting the affected chromosome will manifest the disease, whereas females with one affected X chromosome will be silent (obligate) carriers of the trait (asymptomatic). Carriers do not exhibit clinical bleeding because one allele is capable of producing sufficient, functional F VIIIIC to maintain hemostasis. The ratio of F VIIIIC to the vWF antigen (F VIIIIC/vWF:Ag) should be approximately 1:2 in the carrier.⁵⁶ Occasionally, females may exhibit a mild form of hemophilia due to increased inactivation of the normal X chromosome, known as lyonization. In approximately one-third of newly diagnosed cases of hemophilia A, there may be no previous family history of bleeding. This suggests that a spontaneous mutation may have occurred or that there could be several generations of female silent carriers of the trait.^{51,57}

Patients with F VIIIIC activity less than 1% are classified as severe hemophiliacs. They have a profound hemorrhagic disease, evidenced by intracranial and intramuscular bleeding, hematuria, and spontaneous hemorrhage, requiring daily factor replacement therapy. Frequent, spontaneous hemarthroses are the primary symptom, involving the knees, elbows, ankles, shoulders, hips, and wrists, causing crippling deformities. Moderately severe hemophilia demonstrates F VIIIIC activity between 1% and 5%. These patients have less frequent spontaneous bleeds; however, profuse bleeding still occurs with circumcision, surgery, trauma, or even minor injuries. Activity greater than 5% to 30% is classified as mild hemophilia. These patients may go undiagnosed until a bleeding episode occurs, such as with surgery or trauma. Approximately 10% to 15% of patients with hemophilia A develop antibodies (inhibitors) to F VIIIIC that are usually time- and temperature-dependent on mixing study, and are immunoglobulin (IgG) in nature.⁵⁸ They are capable of destroying F VIIIIC at 37°C, neutralizing the coagulant present in therapeutic infusions and complicating treatment.⁵⁹ Administration of F VIIIIC concentrates may lead to a rise in antibody titers in patients who have already developed antibodies (an anamnestic response) to F VIIIIC (see Circulating Anticoagulants/Inhibitors).⁵³

There are many forms of treatment available for hemophilia A. Concentrates of human plasma F VIIIIC were widely used before 1984. Since then, many patients have been treated with highly purified, heat- or solvent-detergent processed F VIII concentrates, to inactivate hepatitis B, C, and human immunodeficiency viruses (HIV).⁶⁰ Although cryoprecipitate is a rich source of F VIIIIC, it is not a product of choice due to the high incidence of parenteral viral transmission, because antiviral processing is not possible. New technologies that produce synthetic DNA-recombinant F VIII have significantly lowered the incidence of viral transmission. Patients in whom antibodies to human F VIIIIC have developed may often respond to treatment with porcine F VIII; however, they may form antibodies to this product as well. Factor IX concentrate, activated PCC, or *Factor Eight Inhibitor Bypassing Activity*TM (FEIBATM), an anti-inhibitor coagulant complex agent, has been used in severe cases. These products contain factors II, IX, X, activated F VII, and a small amount of antigenic F VIIIIC, which can restore hemostasis by providing factors beyond F VIIIIC to produce thrombin.⁶¹ Thirty percent (0.30 U/mL) F VIIIIC activity is the minimum level required to maintain normal hemostasis.

The laboratory findings for patients with hemophilia A include prolonged aPTT, normal PT, and a normal BT, PFA-100[®]. Mixing studies correct the prolongation of the aPTT. The deficiency is further characterized by F VIIIIC activity under 30%, vWF activity and antigen within the normal reference range, and normal platelet function assays. The presence of an inhibitor can be established when mixing studies fail to correct the prolonged aPTT, particularly after 37°C incubation. An *inhibitor effect* may be present upon factor assay(s), demonstrated as an increase in the apparent activity with each dilutional step (1:10, 1:20, 1:40). The inhibitor measurement is performed using a modified one-stage F VIIIIC method, known as the *Bethesda Assay*; the inhibitor titer is quantified

in Bethesda units (see Specific Inhibitors).⁶² These inhibitors must be differentiated from a nonspecific inhibitor (see Circulating Anticoagulants/Inhibitors) by additional testing and clinical presentation.

Table 27-6 compares hemophilia with classic von Willebrand disease.

Von Willebrand Disease

In 1926, Finnish physician Dr. Erik von Willebrand evaluated a large family from the Åland Islands (an archipelago in the Baltic Sea southwest of Finland) for a severe bleeding disorder. Both sexes were affected and presented with prolonged bleeding times despite normal platelet counts and clot retraction tests.⁶³ Dr. von Willebrand concluded this was a previously undescribed bleeding disorder, and the condition was named in his honor.

vWD differs from classic hemophilia A in three cardinal manifestations: (1) autosomal inheritance rather than sex-linked, (2) consistently prolonged PFA-100[®]s, and (3) mucocutaneous bleeding rather than hemarthroses and deep muscle hemorrhage. It was not until the 1970s that vWF and F VIIIc were found to be different proteins produced by different cells under different genetic control.⁶⁴

vWD is the most common inherited bleeding disorder, affecting both males and females equally. It results from various mutations within the vWF gene located on chromosome 12, causing either quantitative or qualitative defects of plasma vWF. These differences arise due to the characteristics of the mutation(s); the autosomal inheritance pattern, either dominant or recessive; and whether it occurs in a heterozygous or homozygous genotype. These variations in

genotype produce various phenotypes of the disease. vWD is divided into three main types, designated as types 1, 2, and 3. Type 2 vWD is further subdivided into four subtypes, designated as 2A, 2B, 2M, and 2N. There is an additional disease category related to the platelet vWF receptor, known as platelet-type/pseudo-vWD, which has a similar presentation as vWD but does not involve a mutation in the vWF gene. There are also acquired forms of vWD. Each is discussed separately.

Because F VIIIc circulates as a complex with vWF normally in close correlation, patients having reduced or absent levels of the vWF protein may also have reduced or absent levels of F VIIIc. This may initially cause suspicion of F VIIIc deficiency. However, levels of F VIIIc are usually slightly greater than the level of vWF:Ag, presumably because there is a normal gene present for F VIIIc, and the potential binding sites are saturated when levels of vWF:Ag are reduced.⁴⁵ The ABO blood type has also been found to have a significant effect on the amount of vWF:Ag produced; individuals who are type A, B, or AB have much greater mean plasma vWF:Ag concentrations than type O individuals, affecting the severity of symptoms.⁴⁵

Patients with vWD present with mucocutaneous bleeding, frequent epistaxis, ecchymosis, easy bruisability, gastrointestinal bleeding, and menorrhagia, as well as hemorrhage after surgery, childbirth, or dental extractions. These manifestations are caused by the inability of platelets to adhere to subendothelial collagen after injury to the blood vessel.^{65,66} vWF and F VIIIc are acute-phase reactants as well, known to increase during stress, inflammation, pregnancy, with oral contraceptive use, estrogen replacement therapy, or after surgery. These

TABLE 27-6 Comparison of Hemophilia and Classic von Willebrand Disease

Characteristic/Laboratory Result	Hemophilia A	von Willebrand Disease (Type 1)
Deficiency	F VIIIc	vWF
Inheritance	Sex-linked recessive	Autosomal, dominant
Clinical presentation	Hemarthroses; muscle, soft tissue bleeding	Mucosal bleeding: gingival, gastrointestinal, menorrhagia
Bleeding tendency	Moderate to severe	Mild to moderate
Laboratory Tests		
BT, PFA-100 [®]	N	N/ABN (variable)
Platelet count	N	N
RIPA	N	N/ABN (variable)
PT	N	N
aPTT	ABN	N/ABN (variable)
F VIIIc	ABN	N/ABN (variable)
RCA	N	N/ABN (variable)
vWF:Ag	N	N/ABN (variable)
vWF Multimer pattern	N	N pattern with decreased total concentration

N = normal; ABN = abnormal; F VIIIc = factor VIII coagulant activity; PT = prothrombin time; aPTT = activated partial thromboplastin time; vWF:Ag = von Willebrand factor antigen; RCA = ristocetin cofactor assay; PFA-100[®] = platelet function analyzer; RIPA = ristocetin-induced platelet agglutination.

variables create difficulties in the evaluation and diagnosis of patients suspected of having vWD, especially in mild cases. Therefore, it may be necessary to study these patients on multiple occasions to determine whether vWD is the cause of their bleeding symptoms.^{67,68}

Due to the various types of vWD, the clinical presentation can vary. Patients with severe forms of vWD present with a normal PT, prolonged aPTT that corrects on mixing study, normal platelet counts, and abnormal BT, PFA-100® (see Table 27-6). Additional assessments must include assays for F VIII:C activity, vWF activity and antigen, RIPA, LD-RIPA, and vWF multimeric analysis to further identify the specific variant type. This identification is essential for proper diagnosis and treatment (Table 27-7).

Type 1 vWD Type 1, or classic vWD, is the most common type (70% to 80% of cases), inherited as an autosomal dominant trait. It is caused by partial deletions of the vWF gene, leading to a quantitative, proportional deficiency of normal functioning vWF activity and antigen. Symptoms are mild and can present with an abnormal PFA-100® analysis. F VIII:C

activity is decreased, causing the aPTT to be increased. The PT and platelet counts are normal. RIPA may be normal or decreased (dependent on the degree of deficiency), with a normally absent response to LD-RIPA, as well. The multimeric pattern is normal but displays decreased staining intensity (i.e., the distribution of the various molecular weight multimers on agarose gel electrophoresis is complete but equally reduced).

Regular treatment is not required if patients are asymptomatic. Type 1 vWD can be treated prophylactically when necessary with desmopressin acetate, a synthetic analog of the antidiuretic hormone vasopressin, also known as DDAVP (1-deamino-8-D-arginine-vasopressin), before dental work, surgery, and after bleeding episodes, thereby avoiding exposure to blood products. This medication causes the vWF and F VIII:C activity to increase transiently, approximately two- to five-fold, by stimulating release from the endothelial Weibel-Palade bodies. This medication can also be useful in patients with mild hemophilia A. Some patients may not respond to DDAVP; a trial course ("challenge study") may be administered in advance of elective procedures to

TABLE 27-7 Laboratory Diagnosis of Classic von Willebrand Disease (Type 1) and Variants

Laboratory Findings	Type 1	Type 2A	Type 2B	Type 2M	Type 2N	PseudovWD/ Platelet-type	Type 3
Bleeding time	N/ABN (variable)	Marked ABN	ABN	ABN	N/ABN (variable)	ABN	Marked ABN
Platelet count	N	N	N or mild DEC	N	N	Mild DEC	N
F VIII:C	N or DEC	N or DEC	N or DEC	N or DEC	N or DEC	N or DEC	Marked DEC
vWF:Ag	DEC	N or DEC	N or DEC	N or DEC	DEC	N or DEC	Marked DEC
RCA	DEC	Marked DEC	N or DEC	Marked DEC	DEC	N or DEC	Marked DEC
Multimeric Structure of vWF:							
Plasma vWF	N, with quantitative decrease	Absence of intermediate and HMW multimers	Absence of HMW multimers	N; may be larger than normal	N	Absence of HMW multimers	Absent
Platelet vWF	N	Absence of intermediate and HMW multimers	N	N	N	N	Absent
RIPA	N or ABN	Marked DEC	N	ABN	N	N	Markedly decreased
LD-RIPA	N	N	ABN	N	N	ABN	N
F VIII:C-vWF binding	—	—	—	—	DEC	—	—
vWF binding to GP-Ib/IX/V	—	—	INC	—	—	Normal or decreased	—
vWF binding assay	—	—	ABN	—	—	N	—
Spontaneous self-aggregation	—	—	Present	—	—	—	—

N = normal; ABN = abnormal; DEC = decreased; INC = increased; F VIII:C = F VIII coagulant activity; vWF:Ag = von Willebrand factor antigen; PRP = platelet-rich plasma; RCA = ristocetin cofactor activity; RIPA = ristocetin-induced platelet agglutination; LD-RIPA = low-dose ristocetin-induced platelet agglutination; HMW = high molecular weight. Source: From Miller JL. Blood coagulation and fibrinolysis. In Henry JB (ed). Clinical Diagnosis and Management, 17th ed. Philadelphia: WB Saunders; 1984, p 777, with permission.

determine the degree of response. Patients who fail to respond to DDAVP must receive cryoprecipitate or vWF concentrate infusions for prophylaxis or treatment.

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Type 2 vWD The type 2 vWD subtypes (15% to 20% of cases) exhibit qualitative defects of the vWF molecule, with unequal reductions in the components of the F VIII/vWF complex, and abnormalities in the multimeric distribution. They are associated with missense mutations of the vWF gene and inherited as autosomal dominant traits unless stated otherwise.

Type 2A

Type 2A vWD is the most common of the type 2 disorders. A dysfunctional defect of vWF causes a decreased affinity of vWF for platelets, reducing platelet adhesion; this is due to the absence of the HMW multimers in the plasma and platelets. There is a disproportionately lower vWF activity relative to the antigenic concentration, with a ratio generally less than 0.7. Bleeding symptoms may be mild to moderately severe: PFA-100[®], RIPA, and vWF activity are abnormal; platelet count, PT, and LD-RIPA are normal. Levels of F VIIIIC and vWF:Ag may be normal or decreased. The aPTT may be affected, dependent on F VIIIIC levels. Treatment involves the use of vWF concentrates or commercial F VIII concentrates with a near-normal complement of multimers.⁴⁵ DDAVP is ineffective as a treatment modality due to the presence of dysfunctional vWF.

Type 2B

Type 2B vWD exhibits a qualitative defect of vWF in which there is an increased affinity for the platelet GP Ib/IX/V receptor. In this variant, there is an absence of only the high molecular weight multimers in the plasma, whereas the platelet-associated multimers are normal.⁶⁹ The multimers are the most important for normal platelet function; each subunit contains numerous binding sites for the GP Ib/IX/V receptor on resting platelets and the GP IIb/IIIa receptor on activated platelets. This high number of platelet-binding sites correlates with the highly adhesive properties of this fraction, thereby facilitating aggregation.⁷⁰ A differential laboratory finding in this disorder is an abnormal LD-RIPA response ("gain-of-function defect"), that is, enhanced aggregation is seen when testing with very low concentrations of ristocetin (e.g., 0.2 to 0.6 mg/mL). Type 2B vWD requires the use of vWF concentrates; DDAVP administration is contraindicated, as the release of abnormal vWF molecules with increased affinity to normal platelets would cause accelerated clearance, exacerbating the thrombocytopenia.⁴⁵

Type 2M

Type 2M ("multimer") vWD is a very rare form in which the qualitative defect of vWF causes decreased or absent binding of vWF to GP Ib/IX/V as in type 2A; however, the multimer distribution is normal. There are two known

mutations in the GP Ib/IX/V binding site that selectively impair ristocetin-induced binding of vWF to the platelet but do not affect botrocetin-induced binding.⁷¹ This is one of the differential tests that can be used to distinguish this variant. vWF activity is disproportionately decreased relative to levels of the antigen and F VIIIIC, which may be normal or slightly decreased. The binding of vWF to F VIIIIC is normal.⁶⁶ PFA-100[®] is increased with normal platelet counts. The PT is normal, and the aPTT will be normal if the levels of F VIIIIC and vWF antigen are hemostatically normal. The RIPA response is decreased with a normal LD-RIPA. Treatment requires the use of vWF concentrates or cryoprecipitate. DDAVP is ineffective.

Type 2N

Type 2N vWD ("Normandy variant"), initially described in patients from the Normandy region of France, is inherited as an autosomal recessive trait and defined by a markedly decreased affinity of vWF to F VIIIIC. The majority of these patients are either homozygous or compound heterozygous for these mutations; another group of patients with this variant appears to be heterozygous for one of the mutations that produces the F VIIIIC binding defect as well as co-inheriting an allele associated with type 1 vWD.^{69,72,73} The levels of vWF antigen and activity are normal or decreased, the multimer distribution is normal, but the level of F VIIIIC is disproportionately lower than levels of vWF; in the past, this may have caused misdiagnoses of hemophilia. The disorder could be considered an autosomal form of hemophilia A. The platelet-dependent function of vWF is normal; therefore, the RIPA and LD-RIPA will be normal. Platelet count and PFA-100[®] are normal. The F VIIIIC is structurally normal but unstable, with a shortened half-life due to the inability to bind its protective carrier protein. DNA sequence analysis may be used for confirmation in cases where the F VIIIIC binding ability of vWF is severely reduced.⁷⁴ Treatment of this variant requires the use of vWF concentrates or cryoprecipitate. DDAVP is ineffective.

Type 3 vWD Type 3 vWD is the most severe form of quantitative deficiency (1% to 3% of cases), inherited as a homozygous or double heterozygous autosomal recessive trait. Total deletion of the vWF gene causes a complete deficiency of vWF in the plasma, platelets, and endothelial cells. vWF antigen, activity, and multimers are undetectable. F VIIIIC activity is usually 2% to 5% due to the lack of its protective carrier protein.⁷⁵ PFA-100[®], RIPA, and aPTT are severely abnormal. The PT, platelet count, and LD-RIPA are normal. This form may initially be confused with hemophilia A by its clinical presentation (e.g., hemarthroses, intramuscular bleeding). Treatment of this form of vWD requires the replacement of vWF and F VIIIIC, with concentrates that contain a normal complement of multimers and have been treated to inactivate any blood-borne viruses. Development of anti-vWF alloantibodies is possible if patients are infused with plasma-based products, reducing the effectiveness of therapy.⁷⁶ Allo-antibody formation has

been reported in 10% to 15% of type 3 vWD patients as a result of transfusion of vWF-containing products, similar to patients with hemophilia A who produce anti-F VIIIc antibodies (see Specific Inhibitors).⁷⁷

Platelet-Type or Pseudo-vWD Platelet-Type or Pseudo-vWD is not a true vWD; the abnormality is *intrinsic* to the platelet, although clinical bleeding symptoms and laboratory findings are identical to those of type 2B vWD. Classification as pseudo-vWD is due to the absence of mutations in the vWF gene. A mild thrombocytopenia develops, and larger-than-normal platelets are present on a peripheral smear.⁴⁵ The presence of a positive LD-RIPA response, as in type 2B, may cause misdiagnosis. Classification is crucial because treatments differ between these two disorders. Differentiation can be assisted by performance of the vWF binding assay. In this method, the patient's plasma vWF is tagged with a vWF-specific monoclonal antibody and reacted with normal formalin-fixed platelets at various concentrations of ristocetin. This assay cannot rule out pseudo-vWD; a patient with type 2B vWD would show increased vWF binding at lower ristocetin concentrations than normal vWF, or vWF found in pseudo-vWD.⁷⁸ An abnormal vWF binding assay is diagnostic of type 2B vWD. Diagnosis of pseudo-vWD requires a normal vWF binding assay result, in conjunction with other test results typical of this type, as well as vWF and platelet GP Ib/IX/V genetic mutation analysis. Platelet concentrates are required to treat patients with this disorder.

CRITICAL THINKING QUESTION

- 27-2** If hemophilia A and von Willebrand Disease are the result of different deficiencies, why are the PT and PTT results the same for both?

Acquired von Willebrand Syndrome (aVWS) Acquired von Willebrand Syndrome (aVWS) is a very rare bleeding disorder, with symptoms identical to vWD, but without any genetic defect. The aVWS can develop in association with a variety of conditions or without any apparent cause. It can also occur at any age, when there is a negative family history, and either sex is equally affected. Hemorrhagic symptoms vary considerably among patients. The incidence of aVWS is extremely rare. It is most often associated with lymphoproliferative disorders, such as the lymphomas or chronic lymphocytic leukemia, as well as monoclonal gammopathies, such as multiple myeloma and Waldenström macroglobulinemia.

ADVANCED CONTENT

Some cases of aVWS can be eradicated by treating the underlying disorder, but the treatment must be tailored to the specific cause. This may involve surgery, chemotherapy,

radiation, or immunosuppression. The presence of an autoantibody will affect the efficacy of any vWF-replacement product administered to the patient. High-dose intravenous immunoglobulin (IVIG) therapy is effective in these patients. The circulating autoantibody is neutralized by the high content of anti-idiotypic antibodies (i.e., antibodies that interact with the antigen-combining region of other antibodies) in the IVIG preparation.⁷⁹ This allows a spontaneous rise in F VIIIc and vWF levels to occur, which can last 2 to 3 weeks, as prophylaxis for surgical procedures or to maintain hemostasis. However, most cases are managed with supportive measures such as controlling acute hemorrhage or preventing complications from hemorrhage. These measures may include the use of DDAVP, F VIIIc/vWF concentrates, high-dose IVIG therapy, corticosteroids, plasma exchange, immunoadsorption, or recombinant activated F VII concentrates.^{80,81}

Factor IX (Christmas Factor; Plasma Thromboplastin Component [PTC])

Factor IX (F IX) is a single-chain glycoprotein (molecular weight approximately 60 kD) synthesized by the liver and is vitamin K-dependent. The gene coding for its production also resides on the X chromosome. In the coagulation sequence, F IX participates in the intrinsic pathway, where it is activated by F XIa in the presence of calcium ions to become a serine protease, F IXa. A second mechanism of activation, which bypasses the intrinsic contact system (i.e., F XII, high molecular weight kininogen, and prekallikrein), occurs through TF and F VIIa (see Factor VII).⁸² F IXa, in the presence of F VIIIa, calcium ions, and platelet phospholipids (PF₃), forms the *intrinsic tenase complex* that activates F X.⁴³ Although F IXa alone is capable of slowly activating F X in the presence of phospholipid and calcium ions, neither F VIIIc alone nor F VIIIc plus thrombin activates F X in the absence of F IXa.⁸³ Factor IX is as critical to hemostasis as F VIIIc. The generally accepted normal adult reference range is 50% to 150%.

Hemophilia B

Factor IX deficiency, also known as *hemophilia B*, is a sex-linked bleeding disorder, as in hemophilia A. Females with the deficiency are quite rare; they are obligate carriers of the disorder. However, spontaneous mutations do occur. The majority of mutations that cause hemophilia B are missense or nonsense types. There are also deletions or insertion mutations of portions of the gene. It is the type of mutation and the region of the gene affected that determine the clinical severity. The presenting symptoms of hemophilia A and B are identical; it was originally believed that these were the same disorder. In 1947, it was observed that mixing plasmas from two unrelated hemophilic patients would normalize the clotting time in the mixed plasma.^{84,85,86} Hemophilia B became known as "Christmas disease" by Biggs and colleagues,⁸⁷ so named after the surname of the first patient they had seen with the disorder. Since then, F IX is also known as

Christmas factor and synonymously as *plasma thromboplastin component (PTC)* as a necessary factor in the formation of thrombin.

The incidence of inherited F IX deficiency in the United States population is approximately 1 per 30,000, or 100,000 male births worldwide.⁸⁸ As in hemophilia A, the level of F IX activity classifies the severity of the disorder: Severe cases have less than 1% activity; Moderate, greater than 1% to 5% activity; and Mild, greater than 5% to 30% activity. In severe hemophilia B, there is a total absence of F IX both in activity by factor assay, as well as antigenic assay. Due to the variability in mutations, some hemophilia B patients synthesize a nonfunctional or dysfunctional variant of the F IX molecule, whereas others do not have identifiable F IX antigen in the plasma and, therefore, have a true absence of synthesis of the molecule.⁸⁹ Measurement of F IX antigen and activity greatly increases the accuracy of determining carrier status.⁹⁰ Acquired deficiency states can be seen in patients with liver disease, nutritional vitamin K deficiency, and in patients on oral anticoagulant therapy.

Treatment of patients with severe to moderate F IX deficiency consists of infusions of FFP, PCC, or purified F IX concentrates. Patients with mild deficiencies usually receive prophylactic treatment in association with both major and minor surgery. The most serious complication in patients with hemophilia B, as in hemophilia A, is the formation of specific antibodies/inhibitors to F IX. These are extremely difficult to manage, especially in life-threatening situations, and they occur in approximately 10% of patients with F IX deficiency.^{91,92}

The laboratory findings in F IX deficiency include a normal PT, TT, and PFA-100[®]. There is a prolongation of the aPTT that corrects in mixing study. Patients with inhibitors would demonstrate noncorrection of the aPTT. Assays of F IX activity produce decreased to absent levels. Patients with an inhibitor to F IX will be evident as an inhibitor effect during factor assays (as described earlier under hemophilia A); these must be differentiated from a nonspecific inhibitor (see Circulating Anticoagulants/Inhibitors, later in this chapter) by additional testing and clinical presentation.

Factor X (Stuart-Prower Factor)

Factor X (F X) (mw 58.8 kD) is a vitamin K-dependent glycoprotein composed of a light chain and a heavy chain, held together by a single disulfide bond.^{93,94} The heavy chain contains the catalytic domain of the protein.⁹⁵ It is synthesized by the liver and released into the plasma as a precursor to a serine protease. The conversion of F X to its proteolytic form, F Xa, involves the cleavage of a peptide bond in the heavy chain. This reaction, in the intrinsic pathway, is catalyzed by the intrinsic tenase complex (F IXa, F VIIIa, calcium ions, and platelet phospholipid). This same bond is cleaved by the extrinsic tenase complex (F VIIa, TF, and calcium ions) from the extrinsic pathway.⁹⁶

Also known as the Stuart-Prower factor, it gets its name from two cases found to have this abnormality. The first, reported in 1956, was a 22-year-old English woman named Audrey Prower having a history of two significant hemorrhagic

episodes after a dental extraction and tonsillectomy; she also had a brother that died of postoperative hemorrhage after a tonsillectomy at the age of 5.⁹⁷ The second, in 1957, was a 36-year-old male patient named Rufus Stuart.⁹⁴

Congenital F X deficiency is inherited as an autosomal recessive disorder, caused by missense mutations in the gene for F X also carried on chromosome 13.^{98,99} It is extremely rare, with an incidence worldwide of only 1 in 500,000 to 1 in 1 million. Heterozygous patients are asymptomatic and may only be detected during family studies performed after the birth of an afflicted newborn.

Deficiency of F X may occur at any age, but the most severe hemorrhagic symptoms can occur in infancy with excessive umbilical cord bleeding or intracranial hemorrhage. Sites can vary according to the severity of the deficiency. Clinical symptoms range from easy bruising, epistaxis, hematuria, gastrointestinal bleeding, menorrhagia, and after trauma or surgery in mildly affected patients (5% to 10% activity) to hemarthrosis, intramuscular, bleeding central nervous system, and severe postoperative hemorrhage in the most severely affected patients (less than 1% activity). Pregnant women may experience first trimester miscarriage or severe postpartum hemorrhage. The generally accepted normal adult reference range is 50% to 150%.

Usually, the diagnosis of inherited F X deficiency can be made by an appropriate family history and laboratory data. However, differentiating between inherited and acquired deficiencies should include the consideration of liver disease and vitamin K deficiency. Acquired F X deficiencies usually coincide with other vitamin K-dependent factor disorders.

F X deficiencies have been reported in patients with amyloidosis,¹⁰⁰ as well as respiratory infections, acute leukemia, and other malignancies. Rare, variant forms of F X deficiencies exist due to amino acid substitutions or deletions, which produce different patterns of results with PT, aPTT, and F X antigen levels.¹⁰¹

Laboratory testing results typically seen in this deficiency include a prolonged PT and aPTT, both of which correct on mixing study (Table 27-8). The TT and PFA-100[®] are normal. The Stypven time is prolonged, due to its dependence on normal levels of factors II, V, X, fibrinogen, and phospholipid. Factor activity assay, typically using the PT methodology, is decreased or absent. Factor assays by either the

TABLE 27-8 Laboratory Testing and Results for Factor X Deficiency

Test	Result	Reference Range
PT	>14.0 sec	10.0%–14.0 sec*
aPTT	>36 sec	23–36 sec*
F X activity	<50%	50–150%
Stypven time	>25 sec	<25 sec*
Other factor activities	>50%	50%–150%

PT = prothrombin time; aPTT = activated partial thromboplastin time; sec = seconds.

*Genetic reference range for illustrative purpose

PT-based or aPTT-based methods may be used to determine factor activity (F X is part of the common pathway, which is measured by both tests); however, there are variant forms of F X deficiency in which discrepancies in the results can occur, depending on which methodology is used.

Treatment consists of transfusions with FFP or PCCs. The level of F X in PCCs can vary between preparations, and use of these products at higher volumes can lead to thromboses. In 2015, the U.S. Food and Drug Administration approved a human plasma-derived coagulation Factor X concentrate for adults and children over age 12. The need for these treatments should be guided by the severity of the hemorrhagic episode. Additional modes of treatment can also include antifibrinolytic agents for mild bleeding after dental extractions or other minor surgical procedures.

A minimal level of 15% to 20% is considered adequate for hemostasis. If the deficiency is acquired by poor diet or oral anticoagulant therapy, vitamin K supplementation may be therapeutic.

Factor XI (Plasma Thromboplastin Antecedent [PTA])

Factor XI (F XI) (mw 143 kD) is a plasma glycoprotein component of the intrinsic coagulation pathway and is also considered to be part of the contact activation pathway (comprised of F XII, high molecular weight kininogen [HMWK], and prekallikrein [PK]). Synthesized by the liver and secreted into the plasma as a zymogen to a serine protease, it circulates in complex with HMWK.¹⁰² Structurally, it is composed of two identical polypeptide chains linked by a single disulfide bond.^{103,104} In vitro, after contact activation by a negatively charged surface such as glass or reagent silica, celite, or kaolin, F XIa with HMWK acting as a cofactor cleaves the same peptide bond on each chain, forming F XIa. In vivo, there is no physiological activating surface; thrombin activates F XI via a feedback mechanism generated by the TF-F VII pathway. The substrate of F XIa is F IX.

Hemophilia C

F XI deficiency, or **Hemophilia C** (also known as Rosenthal syndrome), is the fourth most common of the inherited bleeding disorders.¹⁰⁵ The estimated incidence in the general population is approximately 1:1 million. It is inherited as an autosomal recessive trait and seen predominately among the Ashkenazi Jewish population in 1:450 individuals.¹⁰⁶ Those most severely affected are homozygous, approximately 0.2%; the heterozygous frequency within this group is nearly 1 in 8.^{107,108}

F XI is the only factor among the contact activation pathway components in which a deficiency may lead to a bleeding diathesis. The remaining proteins participate in the inflammatory response, complement activation, fibrinolysis, and kinin formation.¹¹³ The clinical presentation is very heterogeneous; patients may be asymptomatic, and mild symptoms can range from bruising, epistaxis, menorrhagia, hematuria, prolonged or delayed postpartum bleeding, and bleeding after dental extractions. Those with partial deficiencies can have significant hemorrhagic events requiring massive replacement therapy. Plasma levels do not always predict the occurrence of

postoperative or post-traumatic bleeding. Levels of F XI may fluctuate with time, and bleeding episodes vary in response to a variety of surgical procedures.¹¹¹ There is, however, some degree of correlation between the severity of a hemostatic challenge and severe bleeding.¹⁰³ It has been reported that with F XI deficiency the occurrence of bleeding may correlate with the site of injury or surgical procedure. When a site with high fibrinolytic activity is involved (e.g., the oral cavity, nasopharynx, or genitourinary tract), the risk of bleeding is increased in comparison with sites without fibrinolytic activity.^{109,110}

Normal F XI activity is generally considered to be 70% to 130% and may vary by institution or published reference. Heterozygous individuals have activities that range between 20% and 70%. Homozygous F XI deficient patients may have factor levels ranging from less than 1% activity up to 10%. Antigenic levels parallel the clottable factor activity, demonstrating the decreased level of production of the protein. Levels of less than 20% factor activity are considered severely deficient and will result in postoperative bleeding.

The laboratory features associated with a F XI deficiency include a prolonged aPTT that corrects on mixing study and normal PT. The PFA-100[®] and TT are both normal. F XI activity assays produce decreased or absent levels. Freezing and thawing the plasma can cause cold activation of the contact system and significantly shorten the aPTT in F XI-deficient patients. Therefore, it is advisable to perform the factor activity assay on a fresh plasma sample when there is a high suspicion of deficiency.¹⁰⁴ Because of the variability of factor activity in some individuals, repeat testing is warranted in questionable cases.

Routine replacement therapy is not required for patients with F XI deficiency unless they require surgery. Preoperative infusion of FFP may be required to avoid severe hemorrhage. An increase in factor activity of 20% to 30% is adequate for hemostasis.¹⁰³ Additional treatments can include antifibrinolytic agents, and in patients with known significant bleeding history, plasma-derived F XI concentrates may be used. Recombinant F VIIa has also been used in small dosages for the management of patients undergoing surgery or in patients with inhibitors or having a history of allergy.¹¹⁶ Circulating alloantibodies (inhibitors) can arise in patients with severe F XI deficiency after exposure to plasma products. Further plasma infusions will not control bleeding, but there may be a response to activated PCC (FEIBATM), such as those used to treat patients with F VIII or F IX inhibitors.¹¹²

▶ ADVANCED CONTENT

Recent studies have found that a high level of F XI activity, such as greater than 120%, is a risk factor for venous thrombosis.¹¹⁴ F XI participates in the intrinsic coagulation pathway leading to the formation of thrombin; however, F XI can also be activated by thrombin, both in the presence and in the absence of negatively charged surfaces.¹¹⁵ This feedback mechanism leads to further thrombin generation,

necessary for the formation of fibrin, and for protection against fibrinolysis.¹¹⁶ Thrombin mediates this protective mechanism by activating *thrombin-activatable fibrinolysis inhibitor* (TAFI),¹¹⁷ which removes the sites necessary for binding and activating plasminogen. Therefore, the risk of thrombosis associated with high levels of F XI may be explained by the role of F XI in the inhibition of fibrinolysis.

Factor XII (Hageman Factor)

Factor XII (F XII) (mw 76 kD), also known as Hageman factor, is a single-chain beta globulin.¹¹⁸ The gene has been identified on chromosome 5. It is synthesized by the liver and is one of the factors of the contact activation pathway, circulating as an inactive zymogen. Contact with negatively charged surfaces *in vitro* (solid-phase activation), such as glass, celite, silica, or kaolin, or chemical activation with ellagic acid found in various laboratory reagents, causes the autoactivation of F XII and its conversion to a serine protease.¹¹⁹ This process initiates the intrinsic pathway of coagulation. *In vivo* (fluid-phase) activation occurs by contact with the contents of cell membranes or negatively charged subendothelial structures, such as collagen. In this process, F XII undergoes a conformational change exposing its active site, which then converts PK to kallikrein and subsequently activates F XI.¹²⁰ Additional F XIIa is formed due to cleavage by enzymes such as trypsin, plasmin, or kallikrein. The presence of small amounts of F XIIa leads to the activation of its substrates: PK, F XI, and HMWK. These in turn activate more F XII, amplifying the reactions. F XIIa, F XIa, and kallikrein stimulate the conversion of plasminogen to plasmin, the enzyme critical to fibrinolysis. There is also an integral relationship to the kinin and complement systems with effects toward the inflammatory response.

Deficiency of F XII is a very rare disorder (1:1 million) inherited as an autosomal recessive disorder. It is also known as Hageman trait, after the first patient identified with this deficiency in 1955. Homozygotes are severely deficient (less than 1% activity), whereas heterozygotes may possess 20% to 60% activity. Normal F XII activity is generally considered to be 70% to 140%, varying by institution or published reference. This disorder is asymptomatic; it is not associated with clinical bleeding or surgical/traumatic hemorrhagic risk. Paradoxically, there is an increased risk of spontaneous thromboembolic complications (i.e., pulmonary embolism, myocardial infarction), which could be life-threatening due to inadequate activation of the fibrinolytic pathway. It is notable that Mr. Hageman's death was due to a pulmonary embolism.

This defect is usually an incidental discovery during pre-surgical screening; however, it requires resolution before surgery can proceed. Deficiencies of factors VIII, IX, or XI must be ruled out.

Laboratory analysis indicates a normal PT and a markedly prolonged aPTT, often greater than 200 seconds in homozygotes that corrects on mixing study. Specific factor analysis yields decreased or absent F XII activity, which is required for definitive diagnosis.

No therapeutic factor replacement treatment is required for patients with F XII deficiency. In cases of thromboembolic events, anticoagulation and/or fibrinolytic agents may be required to stabilize the patient. Prophylactic anticoagulation may be instituted in situations where the risk of thrombosis is increased.

Factor XIII (Fibrin-Stabilizing Factor)

F XIII (mw 320 kD) circulates with fibrinogen. In the final stages of the plasma coagulation process, the generation of thrombin, polymerization of fibrin, and the activation of factor XIII (F XIIIa) are responsible for the formation of a stable fibrin clot. F XIII is a zymogen for a plasma transglutaminase; in the presence of fibrin, thrombin and calcium ions convert F XIII to the enzymatic F XIIIa.^{121,122} It is the only enzyme in the coagulation system that is not a serine protease. F XIIIa functions as a catalyst, forming covalent bonds (cross-linkages) between the alpha and gamma chains of fibrin monomers, and various other protein substrates such as alpha-2-antiplasmin, collagen, and fibronectin.¹²³ These actions contribute to hemostasis, proper wound healing, and the maintenance of pregnancy. It has a very long half-life of approximately 1 week. By cross-linking fibrin to alpha 2-antiplasmin, F XIIIa also protects fibrin from fibrinolysis.¹²⁶

ADVANCED CONTENT

Extracellular or plasma F XIII consists of two subunits: two A chains and two B chains in a heterotetrametric conformation. There is also megakaryocyte-synthesized F XIII, primarily found in the platelet alpha granules. The A chains exist in various cells and tissues, such as placenta, platelets, macrophages, and monocytes, and contains the active catalytic site for its transglutaminase activity. The B chains are synthesized in the liver and circulate as free dimers or in complex with the A chains. It is postulated that the B chain contributes to the stabilization of the A chain; however, the function of the B chain remains unknown. Thrombin causes a proteolytic cleavage within the A chain, activating the enzyme; this causes the A and B subunits to become dissociated, exposing the active site on the free A subunits.

Congenital F XIII deficiency is inherited as an autosomal recessive trait and is caused by defects in both F XIIIa and F XIIIb genes; however, the majority of the cases are attributed to genetic variants on the F XIIIa gene, located on chromosome 6.^{124,125}

The prevalence is estimated at 1 in 2 to 3 million population. There is a high frequency of consanguinity in families with this disorder.^{127,128} Clinically, the homozygous state presents with moderate to severe hemorrhagic diatheses, characterized by initial hemostasis followed by the recurrence of bleeding 12 to 36 hours or more after the initial traumatic event (delayed bleeding). This results from the dissolution of the soluble, unstabilized fibrin clot. Soluble fibrin is highly susceptible to degradation by plasmin. This disorder is most often diagnosed

at birth; the most common clinical symptom is bleeding from the umbilical cord stump. Additional symptoms include poor wound healing with abnormal scar formation, recurrent spontaneous miscarriages, bleeding into soft and subcutaneous tissues, and spontaneous CNS bleeding that may ultimately be fatal.

An acquired partial deficiency has been reported with several diseases, including the leukemias, DIC, and severe liver disease. The development of IgG autoantibodies to F XIII has been reported, resulting in severe bleeding diatheses.

Treatments consist of transfusion with FFP or cryoprecipitate that could cause circulatory overload and viral transmission, as well as virus-inactivated plasma F XIII concentrates. There is now a recombinant F XIII concentrate available for the treatment of F XIIIa subunit deficiency.¹²⁵

All routine laboratory screening tests of hemostasis will indicate normal results (i.e., PT, aPTT, fibrinogen, PFA-100®, and platelet count). Historically, the screening test for F XIII deficiency has been the 5 molar urea test, but this has been replaced by chromogenic methods. Antigenic methods include ELISA and automated latex immunoturbidimetry. The normal reference range is approximately 60% to 150%.

Prekallikrein (Fletcher Factor)

Prekallikrein (PK) (approx. mw 100 kD), is a single-chain protein synthesized by the liver.¹²⁹ Conversion to its active form, kallikrein, is catalyzed by F XIIa and HMWK. Approximately 75% circulates bound to HMWK, and 25% circulates as free PK.¹³⁰ Kallikrein, a serine protease, is important in the activation of three other biological systems: the fibrinolytic, complement, and kinin systems. Kallikrein cleaves HMWK into bradykinin, an important mediator in the inflammatory response; it leads to the conversion of plasminogen to plasmin, a potent enzyme that degrades fibrin, fibrinogen, and factors V and VIIIc. Through plasmin, it also activates the complement components C3 and C5. Neutrophils and monocytes are also attracted to the site of injury by the presence of kallikrein (chemoattraction).

Deficiency of PK, known as Fletcher trait (after the family discovered with this disorder), is not associated with clinical bleeding, even during surgery or after severe trauma. It has been reported to be inherited in both an autosomal dominant and recessive trait.¹³¹ The gene resides on chromosome 4. There appears to be no ethnic or racial predilection for this disorder; however, functional and antigenic PK, both absent in Fletcher trait plasma, have been most often reported in black Americans.¹³² Patients with this deficiency have experienced thrombotic events (i.e., myocardial infarction, thromboembolism, and multiple cerebral thromboses), and vascular permeability is also defective.^{133,134}

A marked prolongation of the aPTT is characteristic of this disorder. Mixing studies fully correct the aPTT. In addition, this deficiency can be presumptively identified by extending the contact activation (incubation) time of the patient's plasma with kaolin-like aPTT reagents, beyond the usual 3 to 5 minutes (e.g., 10 minutes and 15 minutes), known as interval incubation, which produces a progressively shorter aPTT. This phenomenon will not occur with other types of factor deficiencies.

Therapy is not indicated for this disorder because there is no hemorrhagic risk. Thromboembolic episodes may require management with anticoagulant therapy.

High Molecular Weight Kininogen (Fitzgerald Factor; Flaujeac Factor; Williams-Fitzgerald-Flaujeac Factor)

High molecular weight kininogen (HMWK) (mw 200 kD) is a single-chain glycoprotein produced by the liver known as the "contact activation cofactor" due to its requirement for surface-dependent activation of F XII and subsequent activation of PK by F XIIa.¹³⁵ Studies indicate that plasma HMWK exists as a procofactor, activated through cleavage by kallikrein.¹³⁶

The kinin system is important in chemotaxis, vascular permeability, and inflammation. Kallikrein cleaves HMWK into a two-chain molecule held by a disulfide bond. This reaction releases bradykinin, a small peptide with actions related to pain, inflammation, increased vascular permeability, and vasodilation. The binding of HMWK to endothelial cells is necessary for the binding of F XI (with which it is in complex) and its activation to F XIa, as well as for the activation of F IX to F IXa by F XIa.¹³⁷

Deficiency of HMWK has been described as an autosomal recessive disorder; and like PK, it shows no predilection to race. HMWK deficiency is rare and not associated with any hemorrhagic diathesis. It presents with a markedly prolonged aPTT that corrects on mixing study. Mild to moderate acquired deficiencies can occur with liver disease or DIC. While there is no associated clinical bleeding disorder, patients with HMWK deficiency have been observed with deep vein thrombosis and pulmonary embolism.¹³⁸

Replacement therapy is not indicated with F XII, PK, or HMWK deficiencies. Patients requiring major surgery have not had any incidence of bleeding. This is presumably because the extrinsic pathway of coagulation, through F VII and tissue factor, remains intact, and F XI can be activated by thrombin generated from the extrinsic pathway.^{139,140} Therefore, F XI can be activated without the need for HMWK, PK, or F XII.

Circulating Anticoagulants/Acquired Inhibitors

Circulating anticoagulants, or acquired inhibitors of coagulation, can develop in patients with certain underlying disorders or spontaneously without any apparent cause. They can have a significant effect on hemostasis and/or interfere with various *in vitro* coagulation tests. Composed of immunoglobulins (IgG, IgM, or IgA), they possess either auto- or alloantibody characteristics.

There are two types of acquired inhibitors that are of major clinical importance: (1) specific inhibitors with a specificity directed toward a single clotting factor and (2) nonspecific inhibitors, also known as the lupus anticoagulant (LA), which interfere with the phospholipid components of the screening reagents, causing a prolongation of the clotting time, therefore suggesting a "state of anticoagulation" or hemorrhagic tendency. When inhibitors are detected, it is

imperative to characterize their specificity; one type can have serious, life-threatening hemorrhagic consequences, whereas the other may cause thrombotic complications that range from mild to severe and life-threatening, or they may be transient and asymptomatic.

Specific Inhibitors

The specific inhibitors are characterized as antibodies. They either directly inhibit (neutralize) the factor's activity or cause increased clearance by forming immune complexes. They may occur secondary to transfusion, replacement therapy, or both. They can also arise in patients with autoimmune disorders or spontaneously in someone without any underlying disease. Inhibitors could potentially arise against any clotting factor.

F VIIIc inhibitors, the most frequently encountered, as well as rare instances of F IX inhibitors, can develop in patients with moderate to severe hemophilia due to transfusion and exposure to the respective deficient factor. These would be classified as *alloantibodies*, antibodies produced against "nonself" antigens. Their presence, however, does not increase the frequency of bleeding episodes.¹⁴⁶ Hemarthroses, muscle and soft tissue hemorrhage, continue to be the clinical symptoms. These F VIIIc inhibitors are predominately IgG antibodies with specificity to the factor's antigenic component of the F VIII/vWF complex but do not interfere with the function of vWF; there is no prolongation of the PFA-100®. An inhibitor should be suspected in any hemophiliac if transfused factor replacement products appear to have reduced effectiveness and/or hemostasis is difficult to achieve.

More frequently, however, inhibitors to F VIIIc may occur as an *autoantibody*, an antibody produced against "self" antigens, which are most often seen in patients with rheumatoid arthritis, systemic lupus erythematosus (SLE) and other autoimmune diseases, drug reactions, and lymphoproliferative diseases or multiple myeloma. They also occasionally occur in women during pregnancy or postpartum and in older adults (>60 years of age) with no apparent underlying disease. This disorder is known as **acquired hemophilia**. Its etiology is unknown and is apparently unrelated to any previous exposure to blood or blood products.¹⁴¹ The clinical symptoms of this disorder may be severe and life-threatening including large hematomas, massive ecchymoses, gross hematuria, retropharyngeal or retroperitoneal hematomas, or cerebral hemorrhage.¹⁴¹ Hemarthroses are less common. The bleeding cannot be controlled with factor replacement and could further exacerbate the condition. Acquired hemophilia should be suspected in anyone without any prior history who presents with massive bruising or hematoma.¹⁴² While the incidence is rare, with approximately 0–2 cases per million per year, the mortality rate can be as high as 10% to 20%.

The classic laboratory findings of a specific inhibitor within the intrinsic pathway, for example, are a normal PT and prolonged aPTT with noncorrection of a mixing study. However, some patients with mild to moderate inhibitors may still show correction. These antibodies also demonstrate a characteristic known as "time-and-temperature dependency"; after a 2-hour 37°C incubation, the patient/pooled normal plasma mixture shows a further prolongation (or worsening of noncorrection)

of the aPTT. This serves to demonstrate the effect of the inhibitor on the factor provided from the pooled normal plasma during the 2-hour period. This step is extremely important to properly characterize the disorder. An "incubation control," consisting of a new mixture prepared from separate, individually incubated patient and pooled normal plasma samples, will not demonstrate the same prolongation. The inhibitor titer can be quantitated in a method known as the *Bethesda Inhibitor Assay* (see Chapter 31).

CRITICAL THINKING QUESTION

27-3 Why is it likely that hemophilia A or hemophilia B patients will develop inhibitors?

ADVANCED CONTENT

Treatment of patients with specific inhibitors is first focused on the stabilization of hemostasis; the overall goal is to eradicate the antibody. The choice of therapeutic modality is dependent on the titer (low versus high) and the patient's response on exposure to additional F VIIIc.¹⁴² Patients with a low titer inhibitor of less than 5 Bethesda Unit (BU) with no increase in titer after exposure to additional F VIIIc (i.e., "low responders") can usually be treated with high concentrations of F VIIIc, in an effort to overwhelm the antibody by saturating all its binding sites. Other options include corticosteroids, porcine F VIIIc, recombinant F VIII concentrates, F IX concentrates, IVIG, and rFVIIa.¹⁴³ Patients with high titer inhibitors (greater than 5 BU) with a marked increase in titer after exposure to F VIIIc (i.e., "high responders") present a treatment dilemma; options can include porcine F VIIIc concentrates; corticosteroids, alone or in combination with immunosuppressive therapy or cytotoxic agents; PCCs; high-dose IVIG; or extracorporeal immunoadsorption or plasmapheresis for patients with very high inhibitor titers.¹⁴² Recombinant F VIIa is the newest therapeutic option available, used primarily to bring hemostasis under control while other modalities focus on suppressing the immune response. Inhibitors can persist for weeks or months; some titers may decline to near zero, only to recur as an anamnestic (memory) response in some patients. Spontaneous remissions have been reported in approximately 38% of cases, most often occurring in women postpartum or in drug-induced reactions.^{141,142}

Spontaneously acquired inhibitors to factors II, VII, and X are very rare. F II inhibitors are described in association with the LA (see Nonspecific Inhibitors). There have been several reports of F XI and F XII inhibitors; several cases were reported as F XI deficiency with acquired inhibitors post-transfusion and other cases occurring in patients with SLE.^{144,145}

Antibodies to vWF can occur in patients congenitally deficient of vWF (severe vWD-type 3) as alloantibodies, as autoantibodies in normal patients, or in those with underlying disorders causing AvWD syndrome (see acquired von Willebrand disease syndrome, earlier in this chapter).

Nonspecific Inhibitors: The Lupus Anticoagulant and Antiphospholipid Antibodies

Nonspecific inhibitors are not directed toward specific factors and are not usually associated with a hemorrhagic risk.^{146,147} They are frequently discovered as an unexpectedly prolonged aPTT on a routine preoperative evaluation. This result would give the impression that the patient may have a factor deficiency. However, mixing studies do not produce a correction of the aPTT. This phenomenon was first recognized in patients with SLE¹⁴⁸ and consequently became known as the **lupus anticoagulant (LA)**. However, this designation is a misnomer because this inhibitor often occurs in patients without SLE who are otherwise healthy and without any underlying medical conditions. Therefore, it would be more appropriate that it be termed a *lupus-like* anticoagulant.

The LA is an immunoglobulin, usually IgG and/or IgM, that interferes with one or more phospholipid-dependent coagulation tests (e.g., aPTT). This laboratory phenomenon is a direct reaction against the negatively charged (anionic) phospholipids present in the reagents. The LA does not inactivate the clotting factors in vitro; it inhibits the formation of the tenase and/or prothrombinase complexes, thereby prolonging the clotting time.^{144,145,149} This in vitro effect of "anticoagulation" is paradoxically associated with an in vivo hypercoagulable (thrombotic) state.

It had previously been discovered that there was a high incidence of biological false-positive results on serological tests for syphilis (VDRL) in SLE patients. This method utilized a phospholipid known as **cardiolipin** (diphosphatidylglycerol) extracted from bovine heart tissue; it is an anionic phospholipid found in the inner mitochondrial membranes of cardiac and skeletal muscle cells and some bacteria. These results were found to be significantly associated with the LA and the risk of thrombosis. It was determined that the sera of these patients contained IgG, IgM, and/or IgA **anticardiolipin (aCL)** antibodies. aCL antibodies and LA both complex with negatively charged phospholipids bound to protein. These **antiphospholipid antibodies (aPL)** each comprise two related but clinically distinct syndromes: the *anticardiolipin antibody (aCL) syndrome* and the *LA syndrome*. Both are frequently associated with thrombosis, fetal loss, or thrombocytopenia, with or without autoimmune disorders.¹⁴² Belonging to a family of disorders known as the **Antiphospholipid Antibody Syndromes (APS)**, they are one of the most common causes of acquired coagulation defects associated with venous and/or arterial thrombosis.¹⁵⁰ The APS can occur spontaneously without any underlying autoimmune disorder, known as *primary APS*. A serious and often fatal manifestation of APS, known as **Catastrophic Antiphospholipid Syndrome (CAPS)**, is

characterized by the development of multiorgan thromboses (infarctions) over a very short period of time (days to weeks). The mortality rate is approximately 50%. *Secondary APS* occurs in association with SLE and other autoimmune diseases, such as acquired immune deficiency syndrome (AIDS), infections of bacterial, viral, or protozoal origin; drugs such as procainamide or chlorpromazine; inflammation; malignancies; and lymphoproliferative disorders.¹⁴²

ADVANCED CONTENT

The LA syndrome is more frequently associated with venous than with arterial thrombosis. The presence of the LA is more specific for APS than the aCL antibody; however, as the titer of aCL antibody increases, its specificity for APS increases, especially if it is of the IgG isotype.¹⁵¹ Approximately 90% of patients with LA have also been found to have at least one isotype of aCL antibody present;^{152,153} however, there are clinical situations in which aCL antibody is present without LA.¹⁵¹ There is also a high incidence of thrombotic disease in patients with elevated aCL antibodies as the titer of antibody increases so does the incidence of thrombosis, fetal loss, and thrombocytopenia.¹⁵⁴ Generally, the presence of aCL antibodies is considered to be a more sensitive indicator than LA in the detection of APS;¹⁵¹ and thromboses associated with aCL syndrome are far more common than with LA syndrome alone.¹⁵⁰ Venous thrombosis presents as deep vein thrombosis (DVT) with or without pulmonary embolism (PE); there may also be thromboses at unusual sites. Arterial thromboses present as strokes (CVA), transient ischemic attacks, or myocardial infarctions.¹⁴²

aPL antibodies are actually directed against negatively charged phospholipid-protein complexes; they require the presence of specific proteins to facilitate phospholipid binding, such as prothrombin, or a natural anticoagulant protein, **beta-2-glycoprotein-1 (β_2 GP-1)**, also known as apolipoprotein H. There have been some cases of patients with LA who have had clinically significant bleeding, which in nearly all cases could be attributed to an abnormality other than the LA.¹⁴² In some cases, the PT is prolonged and may or may not correct with mixing studies. This could be attributed to an acquired hypoprothrombinemia, caused by an antiprothrombin antibody that binds prothrombin but does not neutralize its coagulant activity in vitro; the prothrombin activity and antigen are decreased to the same extent due to immune complex formation and rapid clearance.^{155,156} This has been named *hypoprothrombinemia-LA syndrome*. If the antibody was of a neutralizing type, the prothrombin antigenic concentration would have been normal. Serum antiprothrombin antibodies can be detected by ELISA assays. Other causes of bleeding can be attributed to thrombocytopenia, alone or in combination with a moderate prothrombin deficiency.^{155,157}

Antibodies to β_2 GP-1 are frequently detected in patients with clinical symptoms of APS. Approximately 20% of patients testing negative for aCL antibodies will test positive

for anti- β_2 GP-1.¹⁵⁸ The presence of anti- β_2 GP-1 tends to support a diagnosis of APS in patients with symptoms even though aCL or LA testing may be negative.¹⁵⁹

Laboratory Testing

The ability to detect LA, however, is dependent on the sensitivity of the screening reagents and the preparation of the plasma before analysis. Platelets, rich in phospholipids, must be depleted from the test plasma by centrifugation or filtration to less than 10,000 platelets/ μ L to avoid neutralization of the inhibitor, especially if the sample is to be frozen for testing at a later time; the freeze/thaw cycle lyses the platelets, releasing phospholipids. Hemolysis also poses an interference due to cell lysis; those specimens must be recollected. A patient plasma containing LA usually demonstrates a prolonged (lupus-sensitive) aPTT without correction on mixing study. The PT is usually normal, due to the fact that PT reagents have a much higher phospholipid content, masking the effect. However, it has been observed that up to 30% of LA can initially correct and subsequently exhibit a noncorrection (time-and-temperature-dependent effect) on the incubated mixing study.^{160,161} In addition, aPTT reagents are manufactured with variable sensitivities to the LA; some institutions do not desire a sensitivity to LA, as determined by the medical director, since these usually do not represent a surgical bleeding risk and cause unnecessary delays. The LA may also affect aPTT-based factor assays; a prolongation of the clotting time in factor assays translates to a decreased factor activity. The patient could possibly be misdiagnosed with a factor

deficiency. For this reason, factor assays must be performed with at least two to three serial dilutions to detect a possible "inhibitor effect." The presence of multiple factor assays with an inhibitor effect, noncorrection on mixing studies, and an absence of clinical bleeding evidence are classically suggestive of a nonspecific inhibitor.

Because the LA is a heterogeneous group of antibodies, using only one type of assay is insufficient to diagnose the condition (i.e., no single LA test can detect all positive LA patients or has 100% specificity). The International Society on Thrombosis and Hemostasis (ISTH) recommends samples suspected of having LA be tested using two or more LA screening tests based on different test principles.¹⁶² Screening and confirmation of the LA should include the use of test systems with different types, concentrations, and/or configurations of phospholipids to demonstrate the specificity of the inhibitor (Table 27-9). Phospholipids can be derived from plant or animal sources, or they can be synthetic. These tests should include

- dilute Russell's viper venom time (dRVVT); and
- Platelet phospholipid neutralization procedure (PNP) - high-phospholipid aPTT; and/or
- High-sensitivity aPTT-SCT (Silica Clotting Time); and/or
- Hexagonal Phase Phospholipid Neutralization Test

These are the most sensitive assays currently in use. Other assays with less specificity have included the kaolin clotting time (KCT) and the dilute prothrombin time (dPT) (also known as the tissue thromboplastin inhibition test [TTI]) (see

TABLE 27-9 Screening Tests for Lupus Anticoagulants: Decreased Phospholipid

Test	Nature of Phospholipid	Other Features	Sensitivity	Heparin	Oral Anticoagulant	Factor Deficiency	Specific Inhibitors	Comment
Dilute Russell's Viper Venom Time "Screening" (dRVVT)	Cephalin (phosphatidyl-ethanolamine) Low concentration	Part 1 of 2 reagent system; Common pathway-based reaction	Most sensitive compared with aPTT	Antiheparin agent included in reagent; neutralizes therapeutic dosages	False positive; mixing study required; Confirmatory step may be necessary	False positive with factor II, V, or X deficiency	False positive with factor II, V or X inhibitor	Correction with pooled normal plasma indicates LA not present by this method.
Kaolin Clotting Time (KCT)	No added phospholipid; KCT very sensitive to residual platelets	Patient's plasma phospholipid used in reaction; aPTT-based reaction	Presence of platelets will significantly shorten KCT in presence of LA	False positive	False positive	False positive	False positive	Use of platelet-poor plasma will increase sensitivity
Diluted Prothrombin Time (dPT); Tissue Thromboplastin Inhibition Time	Diluted thromboplastin reagent (PT) with 2 conc. of phospholipid	Protocol similar to dRVVT; 2 step reagent system	Sensitive but not specific; false positive in 30% normal patients	False positive	False positive	False positive with factor II, V, VII, X deficiency	False positive with factor II, V, VII, X inhibitor	May be negative with drug induced IgM LAC

Source: From Triplett DA, Brandt JT. Confirmatory test for lupus anticoagulants. *Hematol Pathol*. 1988;2:121, with permission.

Tables 27-9 and 27-10). These tests may still be used in situations where the clinical suspicion is high despite negative results with the previously described tests; however, they are not recommended by the ISTH, as performing more than two screening tests can lead to a false-positive diagnosis of LA.

Dilute Russell's Viper Venom Time (dRVVT) The dRVVT is an integrated test. Russell's viper venom, an extract from the venom of the Russell's viper (*Daboia russellii*, formerly *Vipera russellii*), directly activates F X, proceeding to fibrin formation via the common pathway. In the first step, the screening reagent contains diluted venom, calcium chloride, and a low concentration of phospholipid as the cofactor for prothrombinase production. This increases the sensitivity to inhibitors of the prothrombinase complex and results in a prolonged clotting time if present. A mixing study performed with this reagent will correct if there is a factor deficiency of the common pathway or if the patient is on warfarin therapy. In the second step, the confirmation reagent contains the same diluted venom and calcium chloride but with a much higher concentration of the same phospholipid. This will neutralize (bypass) the inhibitor by providing additional phospholipid for prothrombinase assembly, resulting in a shorter clotting time. A ratio is calculated for both the screening and confirmation tests by comparison to the mean normal clotting times validated for each reagent. Then, a final "normalized ratio" is determined (i.e., the screening ratio divided by the confirmation ratio). This is used to determine the presence/absence of LA. In general, a normalized ratio greater than 1.2 suggests

LA is present, and less than or equal to 1.20, LA is absent (see Table 27-9).

The commercially available dRVVT reagents contain an antiheparin agent to prevent interferences by this frequent anticoagulant up to therapeutic levels. In addition, this test can be performed on patients with a normal aPTT (as it can vary in its sensitivity to LA); the dRVVT is a more sensitive test than the aPTT. It has also been shown by a number of investigators to be sensitive to β_2 GP-I-dependent antibodies and to correlate very strongly with thrombosis.¹⁶³

Platelet Phospholipid Neutralization Procedure (PNP) The PNP-high-phospholipid aPTT method is a modified aPTT in a two-step process. The phospholipid is an extract derived from human platelets. The first step is an aPTT performed on a mixture of equal volumes of test plasma and saline, producing a prolonged aPTT. The second step is an aPTT performed on a mixture of equal volumes of test plasma and platelet extract. This increases the phospholipid content, significantly shortening the aPTT if LA is present. The difference between the two clotting times is used as the cut-off, determined in studies by the performing laboratory. In addition, the patient's aPTT must be abnormal before this test can be performed to demonstrate the effect of the added phospholipid. False-positive tests may occur in heparinized patients due to platelet factor-4, a potent natural anti-heparin substance contained in the platelet granules. Patients with factor deficiencies of the intrinsic and common pathways would show equal prolongations in both steps.

TABLE 27-10 Confirmatory Tests for Lupus Anticoagulants: Increased Phospholipid*

Test	Nature of Phospholipid	Other Features	Sensitivity	Heparin	Oral Anticoagulant	Factor Deficiency	Specific Inhibitors	Comments
Dilute Russell's Viper Venom Time Confirmation (dRVVT)	Cephalin (phosphatidyl-ethanolamine) High concentration	Part 2 of 2 reagent system; common pathway-based reaction	Most sensitive compared with aPTT	Antiheparin agent included in reagent; neutralizes therapeutic dosages	False positive; mixing study required	—	—	Ratio determination: eliminates interference of factor deficiency
Platelet Phospholipid Neutralization Procedure (PNP)	Human platelet extract	Use with sensitive aPTT reagent system	Sensitive in most cases with aPTT in abnormal range	False positive	—	False positive with factor V deficiency	Weak factor V inhibitor may be false positive	—
Hexagonal Phospholipid-aPTT; Staclot-LA®	Phosphatidyl-ethanolamine	LAC-sensitive aPTT reagent provided	Hexagonal configuration improves sensitivity	Antiheparin agent included in reagent; neutralizes therapeutic dosages	Pooled normal plasma provided in reagent kit; eliminates effect of factor deficiency	Pooled normal plasma provided in reagent kit	Possible false positive with factor VIII inhibitors	—

Source: From Triplett DA, Brandt JT. Confirmatory test for lupus anticoagulants. *Hematol Pathol*. 1988;2:121, with permission.

High-Sensitivity aPTT The high-sensitivity aPTT-silica clotting time (SCT) is an integrated test with two components similar to the dRVVT. In the first step, the screening reagent is a high-sensitivity aPTT reagent containing a colloidal silica activator with a low concentration of phospholipid as the cofactor for intrinsic tenase and prothrombinase assembly. This increases the sensitivity to inhibitors of these enzymatic complexes within the intrinsic and common pathways and results in a prolonged clotting time if present. A mixing study performed with this reagent will correct if there is/are factor deficiencies of these pathways or if the patient is on warfarin therapy. In the second step, the confirmation reagent contains the same colloidal silica activator but with a much higher concentration of the same phospholipid. This will neutralize (bypass) the inhibitor, resulting in a shorter clotting time. A ratio is calculated for both the screening and confirmation tests by comparison to the mean normal clotting times validated for each reagent, producing a normalized ratio. In general, if this ratio is greater than 1.16, it is considered positive for the presence of LA.

Hexagonal Phase Phospholipid Neutralization Test Methodologies using altered phospholipid configurations (i.e., hexagonal phospholipids) have also been used as confirmatory techniques. Changing the epitope (antigenic determinant) to which the antibody may be directed increases the odds of detection (see Table 27-10). Altered configurations display different antigenic determinants than lamellar, or bilayer, molecular structures. This method is similar to the PNP principle of a two-step aPTT; however, it utilizes an LA-sensitive aPTT reagent, phosphatidylethanolamine (PE) as the hexagonal phospholipid, and pooled normal plasma as part of the assay system, simultaneously eliminating the effects of a factor deficiency. A difference of about 8 seconds or more between the test without PE minus the test containing PE is suggestive of LA presence. This assay also contains an antiheparin agent up to therapeutic levels.

The continuing association between aPL antibodies and thrombotic episodes has supported advances in the diagnosis

of this syndrome. The most recent guidelines for LA testing were developed by the International Society on Thrombosis and Hemostasis (ISTH), Scientific and Standardization Committee (SSC), Subcommittee for the Standardization of Lupus Anticoagulant/Antiphospholipid Antibodies. The criteria for the laboratory diagnosis of LA are as follows:

1. The prolongation of at least one phospholipid-dependent clotting test (e.g., aPTT, dRVVT);
2. Evidence of inhibitor activity shown by the effect of patient plasma on pooled normal plasma (noncorrection on mixing study);
3. Evidence that the inhibitor activity is dependent on phospholipid (dRVVT, PNP, SCT, Staclot-LA®, etc.);
4. It must be distinguished carefully from other coagulopathies that may give similar results or occur concurrently with LA (exclusion of specific factor inhibitor).¹⁶⁴

Treatment of thrombosis in patients with APS involves long-term anticoagulation (greater than 6 months to lifelong). If the presence of antibodies resolves, treatment should continue for at least 6 months to ensure no recurrence of the antibodies. Heparin therapy is instituted in a hospital setting as treatment after a thrombotic event, to prevent extension of the existing thrombus or additional thrombosis, until transition to warfarin therapy is in the therapeutic range (INR 2.0 to 3.0; see Chapter 29). Other options can include low molecular weight heparin (injectable, expensive; useful during pregnancy) or low-dose (baby) aspirin therapy in asymptomatic patients as prophylaxis against thrombosis. Newer oral direct thrombin inhibitors (e.g., dabigatran) or oral antifactor Xa inhibitors (e.g., rivaroxaban, apixaban, edoxaban) are available that do not require routine monitoring as with warfarin.

Acknowledgment

The author gratefully acknowledges the contributions of Judith Brody, MD.

SUMMARY CHART

- Screening tests for coagulation abnormalities include:
 - Complete blood count (CBC), platelet count, differential smear
 - Prothrombin time (PT)
 - Activated partial thromboplastin time (aPTT)
 - Fibrinogen
 - Thrombin time
 - PFA-100
- When the platelet count and morphology are normal, the results of the PT and aPTT should be evaluated as follows:
 - For an abnormal PT with a normal aPTT, a mixing study of the PT should be performed using pooled

normal plasma; if the PT is corrected, a factor VII deficiency should be considered, and subsequent factor assay is indicated.

- For an abnormal PT that does not correct with a mixing study, inhibitors to factors II, V, VII, or X should be considered.
- For a normal PT with an abnormal aPTT, a mixing study of the aPTT should be performed using pooled normal plasma. If the aPTT is corrected, a deficiency of factor(s) VIII, IX, XI, XII, prekallikrein (PK) and/or high molecular weight kininogen (HMWK) should be considered, with subsequent testing by specific factor assay performed.

Continued

SUMMARY CHART—cont'd

- For abnormal aPTTs that do not correct with a mixing study, inhibitors to factor(s) VIII:C, IX, XI, or XII should be considered as well as the presence of the lupus anticoagulant or the anticoagulant heparin.
- The presence of heparin can be ruled out if the thrombin time is normal.
- The thrombin time assesses thrombin-fibrinogen interactions and fibrin polymerization. It will be abnormal in the presence of heparin, afibrinogenemia, hypofibrinogenemia, dysfibrinogenemia, and fibrinogen/fibrin degradation products (FDPs).
- A reptilase time will be normal in the presence of heparin.
- For specimens that have both an abnormal PT and aPTT that corrects with mixing studies, deficiencies of factors I, II, V, and X should first be considered.
- If an abnormal PT and aPTT does not correct with mixing studies, an inhibitor to factors II, V, or X should be considered.
- Laboratory findings in patients with hemophilia A include a prolonged aPTT, normal PT, and normal bleeding time; mixing studies will correct the aPTT.
- Laboratory studies for von Willebrand disease should include a bleeding time, platelet count, aPTT, mixing study, F VIII:C activity, von Willebrand factor antigen (vWF:Ag), von Willebrand factor activity, RIPA, LD-RIPA, and multimer analysis.
- The most common inhibitor to a specific clotting factor is factor VIII:C (F VIII:C); the inhibitor is predominantly IgG, and time and temperature-dependent.
- A deficiency in F VIII results in hemophilia A.
- A deficiency in F IX results in hemophilia B.
- A deficiency in F XI results in hemophilia C.
- The vitamin K-dependent factors are F II, F VII, F IX, and F X.
- The lupus anticoagulants may be IgG, IgM, or IgA in nature and can be detected by: dilute Russell's viper venom time (dRVVT), platelet phospholipid neutralization procedure (PNP)-high-phospholipid aPTT, and/or high-sensitivity aPTT-SCT (silica clotting time), and/or hexagonal phase phospholipid neutralization test.

CASE STUDY 27-1

A 12-year-old boy underwent tooth extraction in preparation for orthodontia. Persistent bleeding followed. The history was remarkable for bruising and frequent epistaxis. The patient's mother also experienced easy bruising and menorrhagia. Physical examination revealed several medium-sized ecchymotic lesions on the lower extremities. Laboratory findings were as follows:

Test	Result	Ref. Values
PT	12.0 sec	10.0–14.0 sec
APTT	40 sec	23–36 sec
PFA-100*	Prolonged	Normal Closure Time (ADP/Collagen)
Platelet count	$300 \times 10^9/L$	$150\text{--}450 \times 10^9/L$
F VIII:C	30%	50%–150%
vWF:Ag	45%	50%–150%
vWFR:Co	41%	50%–150%
RIPA*	Depressed response	Normal response
Multimers	Normal	Normal

*Ristocetin-induced platelet aggregation.

The history of bruising, epistaxis, and dental bleeding with a prolonged aPTT, PFA-100*, and reduced F VIII:C,

vWF:Ag, vWFR:Co, and RIPA are indicative of classic von Willebrand disease (type 1).

QUESTIONS

1. Which blood product could correct the RIPA value in this case?
2. Which laboratory values reflect a type 1 von Willebrand disease?
3. What form of therapy would be indicated here?

ANSWERS

1. Concentrates of F VIII:C, cryoprecipitate, and von Willebrand factor can correct the RIPA response in this case.
2. Reduced levels of F VIII:C (30%), vWf:Ag (45%), and vWfR:Co (41%) along with the prolonged bleeding time and depressed RIPA are classically seen with type 1 von Willebrand's disease.
3. DDAVP would be advantageous as a prophylactic treatment before any future dental extractions or minor surgical procedure. This avoids exposure to any blood products by causing a transient increase in F VIII:C and vWF.

CASE STUDY 27-2

A 5-year-old boy presented in the emergency department with a hemarthrosis of the right knee after falling off a playground swing. Physical examination revealed a well-nourished child with no fractures or other ecchymoses. He was afebrile. Laboratory findings were as follows:

Test	Result	Ref. Values
CBC and platelet count	Normal	Normal
PT	13.0 sec	10.0–14.0 sec
aPTT	87 sec	23–36 sec
Fibrinogen	325 mg/dL	200–400 mg/dL
PFA-100	Normal	Normal

Further studies included:

aPTT mixing study	32 sec	23–36 sec
FVIII:C	10%	50%–150%
vWF:Ag	90%	50%–150%

This patient was known to have hemophilia A.

QUESTIONS

1. What material did the laboratory professional use when performing this mixing study?
2. What treatment is indicated for this patient?

ANSWERS

1. The mixing study is performed with pooled normal plasma.
2. This patient requires infusion of factor VIII concentrate.

CASE STUDY 27-3

A 40-year-old woman was scheduled for an elective surgical procedure. Preadmission testing revealed the results below:

Test	Result	Ref. Values
CBC and platelet count	Normal	Normal
PT	18.0 sec	10.0–14.0 sec
aPTT	30 sec	23–36 sec

After further examination, the patient stated that she had episodes of epistaxis and easy bruising. Additional laboratory testing included:

PT mixing study	13.0 sec	10.0–14.0 sec
FVII	30%	50%–150%

This patient has F VII deficiency. F VII is not measured by the aPTT and is normal, whereas the PT is sensitive to deficiencies of the extrinsic system (factors I, II, V, VII, and X) and is prolonged in these cases.

QUESTIONS

1. What material is used to perform the mixing study?
2. What treatment is indicated here?
3. Why was the PT prolonged and the aPTT normal in this case?

ANSWERS

1. The mixing study is performed with pooled normal plasma.
2. This patient would require fresh frozen plasma or prothrombin-complex concentrate. However, if the deficiency can be attributed to poor diet, liver disease, or warfarin therapy, vitamin K supplementation may be preferable to using blood products.
3. The PT was prolonged because the extrinsic pathway could not proceed normally, caused by the deficiency of F VII. The aPTT was normal because F VII does not participate in the intrinsic pathway and therefore the test is not sensitive to this defect.

CASE STUDY 27-4

A 29-year-old woman was seen by her obstetrician for prenatal care. She had three previous pregnancies, two resulting in spontaneous first-trimester miscarriages and the third in fetal demise at 28 weeks' gestation. Laboratory values were as follows:

Test	Result	Ref. Values
CBC and platelet count	Normal	Normal
PT	11.5 sec	10.0–14.0 sec
aPTT	45 sec	23–36 sec
Fibrinogen	375 mg/dL	200–400 mg/dL

Further studies included:

aPTT mixing study	43.0 sec	23–36 sec
DRVVT ratio	1.60	<1.20
PNP difference	15 sec	<5 sec
Anticardiolipin Ab	positive	Negative

This patient has a lupus anticoagulant with positive anticardiolipin antibodies. These are known to cause recurrent spontaneous abortion, as well as thrombotic events such as stroke, deep vein thromboses, and pulmonary embolism.

CASE STUDY 27-5

A 60-year-old woman was admitted to the emergency department with hemorrhage into the right arm and right breast. No previous history of bleeding or medication was indicated. Laboratory data upon admission were as follows:

Test	Result	Ref. Values
PT	12.0 sec	10.0–14.0 sec
aPTT	58 sec	23–36 sec
Fibrinogen	400 mg/dL	200–400 mg/dL
aPTT mixing study	40 sec	23–36 sec
DRVVT ratio	1.1	<1.2
PNP difference	1 sec	<5 sec

This patient does not have a lupus anticoagulant but still does not correct in a mixing study with pooled normal plasma. Further studies must be performed. Incubation of the patient's plasma with pooled normal plasma for 2 hours at 37°C demonstrates time- and temperature-dependency, indicating the presence of an inhibitor. In addition, factor assays indicate decreased activity of a single factor.

F VIIIc	10%	50%–150%
F IX	90%	50%–150%
F XI	95%	70%–130%

QUESTIONS

1. What complex is inhibited by LA, causing a prolongation of the clotting time?
2. Why does the PNP show a significant difference in this patient?
3. What form of treatment is indicated here?

ANSWERS

1. The complex that is inhibited by LA is the prothrombinase complex (factors Xa, V, II, calcium, and phospholipid), delaying the formation of fibrin and producing a prolonged clotting time.
2. The high concentration of phospholipid in the PNP bypasses or "neutralizes" the inhibitor thereby creating a greater difference versus the aPTT without the additional phospholipid.
3. Treatment of a patient with LA would require anticoagulation with warfarin or, in some circumstances, heparin may be used (e.g., during pregnancy).

Factor inhibitor analysis (Bethesda assay) for F VIIIc produced a titer of 8 Bethesda units of inhibitor activity (normal is less than 0.5 units). One Bethesda unit of inhibitor is equivalent to 50% residual factor activity after incubation at 37°C for 2 hours. This patient developed a spontaneous inhibitor to F VIIIc that resulted in the massive bleeding into the tissue of her arm and breast.

QUESTIONS

1. Which test(s) rule out the presence of a lupus anticoagulant?
2. What treatment is indicated here?

ANSWERS

1. The presence of a lupus anticoagulant is ruled out by the negative DRVVT and PNP tests.
2. Treatment of a F VIIIc inhibitor can include porcine F VIIIc concentrates; prothrombin-complex concentrates; steroids (alone or in combination); immunosuppressive therapy; cytotoxic agents; high-dose intravenous immunoglobulin, immunoadsorption or plasmapheresis if the inhibitor titer is very high.

REVIEW QUESTIONS

1. Impaired hemostasis due to clotting factor defects can be caused by:
 - a. Decreased factor synthesis
 - b. Decreased platelet concentration
 - c. Loss, consumption, or inactivation of platelets
 - d. Abnormalities of the blood vessels
2. Which following disorder refers to decreased activity of F VIIIc?
 - a. Hemophilia A
 - b. Hemophilia B
 - c. Parahemophilia
 - d. Hemophilia C
3. Which coagulation disorder has decreased activity of F VIIIc, vWF:Ag, and vWF activity, and a prolonged PFA-100 test?
 - a. Thrombocytopenia
 - b. von Willebrand disease
 - c. Hemophilia A
 - d. Acquired hemophilia
4. The factor deficiency that produces a normal aPTT and an abnormal PT is:
 - a. Afibrinogenemia
 - b. Prekallikrein
 - c. Factor VII
 - d. Factor X
5. A patient with amyloidosis can have a deficiency of which factor?
 - a. Factor V
 - b. Factor X
 - c. Factor VIIIc
 - d. Factor II
6. The reptilase time will be normal in the presence of:
 - a. Heparin
 - b. Dysfibrinogenemia
 - c. Afibrinogenemia
 - d. Hypofibrinogenemia
7. Which of the following is a test for identification of a lupus anticoagulant?
 - a. Platelet neutralization procedure (PNP)
 - b. PT
 - c. Platelet count
 - d. Thrombin time
8. All of the following statements are true regarding the detection of a lupus anticoagulant (LA) except:
 - a. Platelet-poor plasma samples are required.
 - b. The aPTT is frequently prolonged and is usually the first indication of an LA.
 - c. A mixing study will correct the prolonged aPTT.
 - d. The dilute Russell's viper venom time may be used to confirm the presence of LA.
9. Christmas factor is another name for which of the following factors?
 - a. F II
 - b. F IX
 - c. F XI
 - d. F X
10. Clotting factor deficiencies are more likely to present as:
 - a. Purpura
 - b. Venous bleeding disorders
 - c. Mucosal bleeding
 - d. Superficial bruising
11. Which of the following is a nonspecific inhibitor?
 - a. F VIIIc inhibitor
 - b. F IX inhibitor
 - c. F II inhibitor
 - d. Lupus anticoagulant

See answers at the back of this book.

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Disseminated Intravascular Coagulation and Primary Fibrinolysis

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CHAPTER OUTLINE

Components of the Fibrinolytic System

Plasminogen
Plasminogen Activators
Plasminogen Activator Inhibitor-1
Plasmin
 α_2 -Antiplasmin
Thrombomodulin
Thrombin-Activatable Fibrinolysis Inhibitor
Fibrin and Fibrinogen

Congenital Abnormalities of the Fibrinolytic System

Disseminated Intravascular Coagulation
Triggering Mechanisms and Associated Clinical Disorders
Clinical Presentation
Laboratory Diagnosis
Treatment

Related Disorders

Summary Chart
Case Study 28-1
Review Questions
References

LEARNING OBJECTIVES

At the end of this chapter, the learner will be able to:

- 28-1 Describe the purpose of the fibrinolytic system.
- 28-2 Evaluate the components of the coagulation and fibrinolytic systems.
- 28-3 Describe the function of the proteolytic systems involved with fibrinolysis.
- 28-4 Explain the three mechanisms that activate plasminogen.
- 28-5 Identify the consequences of fibrinolytic system abnormalities.
- 28-6 Recognize the conditions that can trigger disseminated intravascular coagulation.
- 28-7 Assess the clinical presentation of acute and chronic disseminated intravascular coagulation.
- 28-8 Compare the laboratory results found in acute and chronic disseminated intravascular coagulation.
- 28-9 Examine therapies used to treat both acute and chronic disseminated intravascular coagulation.
- 28-10 Compare the laboratory abnormalities associated with primary fibrinolysis, thrombotic thrombocytopenic purpura, and disseminated intravascular coagulation.

Normal hemostasis is the result of the balanced interaction of the vascular endothelium and platelets with four biochemical systems: the coagulation, the fibrinolytic, the kinin, and, to a lesser extent, the complement systems. The interrelationship between these systems is illustrated in Figure 28-1. The endothelium provides procoagulant, fibrinolytic, anticoagulant, and inflammatory functions, but these functions vary among the different vascular beds and may be organ specific in some cases.¹

When a stimulus initiates activation of the coagulation system, a series of enzymes comprising the fibrinolytic system are activated to lyse the fibrin, reestablishing vessel lumen integrity and blood flow. This chapter describes the biochemistry components of the fibrinolytic system, its associated pathophysiologic disorders, and laboratory tests

available to evaluate individual components and overall function.

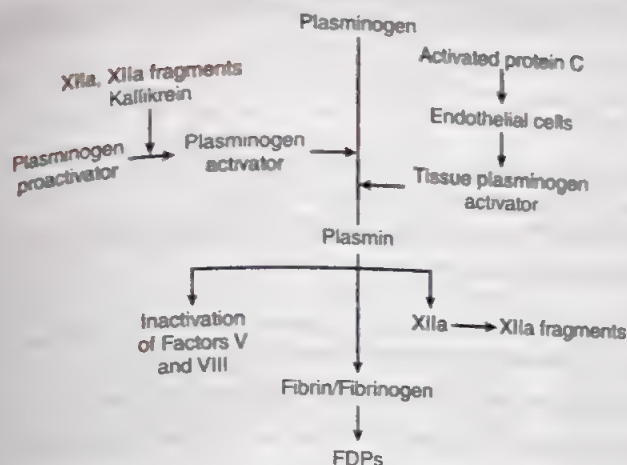
Components of the Fibrinolytic System

The components of the fibrinolytic system are included in Table 28-1.

Plasminogen

Plasminogen is a single-chain plasma zymogen of approximately 90 kD that circulates in two molecular forms, differing only in their carbohydrate content.² Each form circulates in human plasma in equal amounts. It is synthesized by the liver and has a half-life of 2 days. The plasma content is approximately 20 mg/dL. Each form of this molecule has an amino acid terminal glutamic acid (Glu-plasminogen) and

FIBRINOLYTIC SYSTEM



COAGULATION SYSTEM

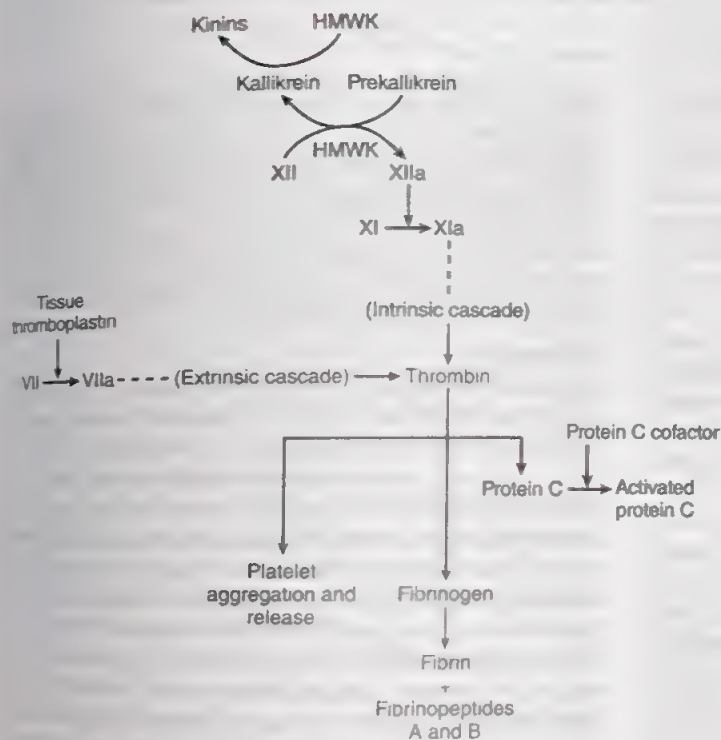


FIGURE 28-1 Summary of the interaction of the coagulation, fibrinolytic, and kinin systems. High molecular weight kininogen (HMWK) and prekallikrein catalyze the activation of factor XII to factor XIIa. Factor XIIa then promotes the conversion of prekallikrein to kallikrein. The latter liberates the kinins from HMWK, thus completing the positive feedback loops of the contact phase of coagulation. These components stimulate endothelial cells to release plasminogen activator. Thrombin generated through the extrinsic and intrinsic coagulation cascades then converts fibrinogen to fibrin, induces platelet activation, activates protein C, and stimulates both tissue plasminogen activator (tPA) and urokinase-like plasminogen activator (uPA) from the endothelium, which then can convert plasminogen to plasmin. Thrombin exerts a negative feedback by simultaneously stimulating plasminogen activator inhibitor 1 (PAI-1) release from the endothelial cells. PAI-1 will serve to bind tPA and uPA to dampen plasmin generation. Plasmin, once formed, will initiate clot lysis by proteolytically cleaving fibrin and fibrinogen into fibrin/fibrinogen degradation products (FDPs). In addition, plasmin will inactivate a number of coagulation factors including factors V and VIII and convert activated factor XII into XIIa fragments. Excess plasmin in circulation is prevented by the presence of α_2 -antiplasmin, which forms a complex with plasmin, thus inactivating it.

can undergo limited proteolytic cleavage of this region to an incomplete molecule with lysine as the new terminal amino acid (Lys-plasminogen).

Plasminogen Activators

The conversion of plasminogen to active plasmin can occur via a group of activating proteins collectively known as *plasminogen activators*. Regardless of the initiating mechanism, activation of plasminogen to yield plasmin proceeds through the cleavage of the same arginine 560–valine 561 bond in the Glu and Lys forms of plasminogen.

The major activators originate primarily in the vascular endothelium, the site of synthesis of both tissue plasminogen activator (tPA) and endothelial cell urokinase, a single-chain precursor. Urokinase plays a minor role in fibrinolysis but is an important activator within the genitourinary system. Single-chain urokinase is converted to its active form, two-chain urokinase, by either plasmin or kallikrein and is rapidly inhibited by plasminogen activator inhibitor-1 (PAI-1).

Intrinsic activators are serine proteases present in the blood and a variety of other tissues, particularly the vascular endothelium. With the initiation of the contact phase of

TABLE 28-1 Components of the Fibrinolytic System

Component	Description
1. Plasminogen	Circulating inactive plasma protein produced in liver
2. Plasminogen activators:	
• Tissue plasminogen activator (tPA)	Extrinsic activator liberated from endothelial cells by the action of thrombin
• Urokinase	Secreted by epithelial cells, monocytes, and macrophages
• Streptokinase	Bacterial cell product; forms a complex with plasminogen that has activating activity
3. Plasminogen activator inhibitor-1 (PAI-1)	Inhibitor of tissue plasminogen activator and urokinase
4. Plasmin	Active serine protease of 70–75 kD
5. α_2 -Antiplasmin	Primary inhibitor of plasmin; forms an irreversible complex with plasmin
6. Thrombomodulin	Endothelial cell membrane glycoprotein that serves as a receptor for thrombin
7. Thrombin-activatable fibrinolysis inhibitor (TAFI)	Plasma procarboxypeptidase B, activated by thrombin, which suppresses plasminogen binding to fibrin
8. Fibrin and fibrinogen	Plasmin substrates; proteolytic cleavage results in the generation of degradation products

coagulation (see Chapter 25), factor XIa, XIIa fragments, kallikrein, and high molecular weight kininogen interact to yield plasminogen-activating ability.^{3,4} The exact biochemical steps involved in the formation of this intrinsic activator are not completely understood. The activator activity generated by this pathway slowly converts plasminogen to plasmin.

Tissue Plasminogen Activator (tPA)

tPA is the primary plasminogen activator within the vascular circulation. Its greater efficiency in thrombolytic therapy and potential for pharmacological manipulation have led to its widespread use as a therapeutic fibrinolytic agent. tPA is an endothelial cell product with a molecular mass of approximately 68 kD.⁵

Although several biochemical stimuli, including histamine, vasopressin, bradykinin, and adrenaline can induce tPA release, thrombin is the most important release inducer. Thrombin thus serves as a coagulant component (converting fibrinogen to fibrin and aggregating platelets); as a stimulant to manage the coagulant process by binding to thrombomodulin, thus allowing for the generation of activated protein C; and as an initiator of fibrinolysis by stimulating endothelial cells to release tPA and urokinase (see Fig. 28-1). It is now known that thrombin, in addition to causing endothelial cell release of PAI-1, also acts to inhibit fibrinolysis through a second mechanism. Thrombin works with soluble thrombomodulin fragments to convert procarboxypeptidase B, a plasma protein, to its active form, which proteolytically cleaves the plasminogen binding site (lysine residue) on fibrin and thus prevents plasmin formation and progressive clot lysis. This function is termed *thrombin-activatable fibrinolysis inhibitor (TAFI)*.⁶

Activated protein C exerts a negative feedback control on the coagulation process by proteolytically cleaving activated coagulant factors Va and VIIIa, thus limiting fibrin formation. tPA has a high affinity for fibrin, and its adsorption to fibrin clots greatly enhances plasminogen conversion to plasmin.

Because of a high affinity of both the plasminogen activator and plasminogen for fibrin rather than fibrinogen, the effect of this reaction is accentuated on the surface of and within the clot. Release of tPA from endothelium is also responsive to a variety of other stimuli, including venous occlusion, strenuous exercise, and treatment with vasoactive drugs, such as the vasopressin derivative DDAVP. tPA activity is increased several-fold under these conditions.⁷

Urokinase

Therapeutic exogenous activators have been available for clinical use for several years. As mentioned earlier, tPA is widely used as a therapeutic agent to promote fibrinolysis. Other therapeutic agents include urokinase and streptokinase. Urokinase is synthesized by the kidney and the vascular endothelium, as previously mentioned, and is excreted in the urine.⁸ It can also be identified in vitro using kidney cell cultures and is a potent direct activator of plasminogen. Its major drawbacks are its expense and relatively lower affinity for fibrin compared with tPA. A consequence of the latter property is that the plasmin digests fibrin as well as fibrinogen. Therefore, the development of severe hypofibrinogenemia is not uncommon with its use. The other exogenous activator, **streptokinase**, is a product of beta (B)-hemolytic streptococci.

Streptokinase

Streptokinase is not a serine protease and has no intrinsic proteolytic activity but can form a complex with plasminogen. This interaction results in a conformational change of the plasminogen molecule and exposure of its active serine site.⁹ The streptokinase-plasminogen complex can then undergo autocatalysis to yield other activators such as streptokinase-Glu-plasmin and streptokinase-Lys-plasmin. Any of these forms will readily convert free plasminogen to plasmin. Because streptokinase is a bacterial protein, a major limitation with its use in thrombolytic therapy is the induction of an immune response, with resulting antibody development and inhibition of its activity.

Plasminogen Activator Inhibitor-1

Plasminogen activator inhibitor-1 (PAI-1) is one of the most important inhibitors of the fibrinolytic system and is a member of the family of protease inhibitors that includes antithrombin (formerly known as antithrombin III), α_1 -macroglobulin, α_1 -antitrypsin, and α_2 -antiplasmin, which are collectively referred to as *serpins* (*serine protease inhibitors*). PAI-1 is a 53-kD glycoprotein also synthesized by vascular endothelium and released primarily in an inactive, latent state.¹⁰ It is an acute-phase reactant and can be induced by a variety of stimuli, including interleukin-1 (IL-1), endotoxin, and thrombin. PAI-1 is the primary inhibitor of both tPA and urokinase. Thus, regulation of fibrinolysis is dependent on the interaction of tPA and urokinase with PAI-1. Under basal conditions, most of the tPA released is bound to PAI-1.

Plasmin

The pivotal serine protease generated through these complex biochemical processes is **plasmin**. This protein has a molecular weight of 77 to 85 kD, depending on whether Lys-plasmin or Glu-plasmin is formed, and it has a transient plasma half-life measured in seconds.¹¹ Plasmin can proteolytically degrade both fibrin in clots and native fibrinogen in the circulation into a series of well-characterized end products collectively known as **fibrin/fibrinogen degradation products (FDPs)**. This process results in an asymmetric, progressive breakdown of fibrin and fibrinogen.¹¹

The earliest recognized component is fragment X, which is still capable of clotting. Fragment X consists of two separate D units with an E unit in the middle. The X fragment undergoes further plasmin attack to yield Y (consisting of one D and E unit) and D fragments that are not capable of clotting. The Y fragment is further digested to yield an additional D fragment and a single E fragment.

The proteolytic cleavage of cross-linked fibrin (i.e., fibrin stabilized through the action of factor XIIIa and calcium) results in other intermediate degradation products. This proteolytic product is referred to as the D-dimer, consisting of 2 D units double-bound together.

These breakdown products have specific inhibitory effects on the coagulation system and thereby suppress further clot formation. Fragment X is capable of clotting slowly and exerts an anticoagulant effect by competing with fibrinogen for thrombin. It also forms slowly polymerizing complexes with fibrin monomer and inhibits the polymerization step. Fragment D forms abnormal complexes with fibrin monomers as it polymerizes. Fragment E is not known to have any specific anticoagulant effect. In high concentrations (more than 100 mcg/mL), the degradation products are capable of inhibiting platelet aggregation and release. Plasmin also exerts a direct limiting effect on the coagulation process by being able to proteolytically cleave and render inactive factors V and VIII, in addition to breaking down factor XII and platelet glycoprotein Ib/IX/V, the von Willebrand's factor receptor.

α_2 -Antiplasmin

Plasmin formation characteristically takes place around fibrin deposition with little free plasmin circulating. However,

this plasmin enzyme if unchecked by the presence of specific inhibitors, would result in circulating fibrinogen being digested, decreasing the blood's ability to clot. The primary physiological inhibitor of plasmin *in vivo* is α_2 -antiplasmin.¹² It rapidly binds irreversibly to the lysine binding site on plasmin in a 1:1 molar ratio. Other protease inhibitors in plasma include α_2 -macroglobulin, C1 inactivator, and α_1 -antitrypsin. Of these, only α_2 -macroglobulin has a role in plasmin inhibition during normal hemostasis, but it participates only when α_2 -antiplasmin binding sites for plasmin are saturated.

ADVANCED CONTENT

Measurement of these plasmin- α_2 -antiplasmin complexes has been suggested as an indicator for activation of the fibrinolytic system. Plasmin adsorbed onto fibrin during the fibrinolytic process appears to be protected from this inhibitor because it binds to fibrin through the same lysine binding site. Once the binding site on plasmin is occupied, the inhibitor cannot bind, and clot lysis can proceed. The overall effect is to ensure that plasmin activity is limited to the area of fibrin deposition and to prevent free plasmin from circulating.

Thrombomodulin

Thrombomodulin is an endothelial cell membrane glycoprotein that is a receptor for thrombin and forms a 1:1 complex with thrombin. Thrombin, once bound to thrombomodulin, no longer has proteolytic activity to convert fibrinogen to fibrin but retains its esterolytic function.¹³ The complex serves to activate protein C. Activated protein C forms a complex with free protein S (not bound to complement) and exerts negative feedback control on the coagulation process by proteolytically cleaving and inactivating coagulant factors Va and VIIIa, thus limiting further clot formation.

ADVANCED CONTENT

The thrombin-thrombomodulin complex also serves to convert procarboxypeptidase B to its activated form, thrombin-activatable fibrinolysis inhibitor (TAFI). The number of thrombomodulin sites on the vascular endothelium is downregulated by proinflammatory cytokines including IL-6 and tissue necrosis factor (TNF) and by other inflammatory modulators released from activated neutrophils during the inflammatory process.

Thrombin-Activatable Fibrinolysis Inhibitor

Thrombin-activatable fibrinolysis inhibitor (TAFI) circulates in plasma in an inactive form (zymogen) as procarboxypeptidase B and is converted to its active form by the thrombin-thrombomodulin complex. Carboxypeptidases are enzymes that hydrolyze carboxypeptidase bonds. The protein is synthesized in the liver and has a molecular mass of 53 to

60 kD. TAFI inhibits fibrinolysis by hydrolyzing lysine (lysyl residues) from the carboxyl end of fibrin. These same residues serve as binding sites on the fibrin clot for plasminogen-tPA complex to initiate fibrinogen/fibrin degradation.¹⁴

Fibrin and Fibrinogen

Fibrin is a filamentous and insoluble protein that is responsible for stable clot formation. It is produced by the action of thrombin on fibrinogen, converting it to fibrin. Fibrinogen is a plasma glycoprotein produced in the liver.

CRITICAL THINKING QUESTION

28-1 Why are so many inhibitors and components involved in the fibrinolytic system?

See answers to all Critical Thinking Questions at the back of this book.

Congenital Abnormalities of the Fibrinolytic System

Congenital abnormalities of the fibrinolytic system causing either a hemorrhagic or thrombotic diathesis are rare. Only three cases of an abnormal plasminogen have been reported in which the patients had a history of recurrent thrombotic episodes. In essentially all other cases reported, patients have been asymptomatic even though a hypercoagulable state would have been expected.¹⁵ Plasminogen deficiencies have also been reported in patients that have developed **lignous conjunctivitis** (fibrin-rich pseudomembranes that form on the eyes). Low levels of tPA activity have been documented in two families and were associated with a similar thrombotic tendency. Whether deficiencies of tPA or urokinase cause a hypercoagulable state is controversial. The Quebec platelet disorder, which leads to a bleeding diathesis associated with moderate to severe thrombocytopenia, has been found to be due to excess urokinase content in the α granules of platelets.¹⁶ Excess levels of PAI-1 have been associated with thrombotic disease. A common diallelic polymorphism has been described for PAI-1 in which the prevalence of the 4G allele is significantly higher in patients with myocardial infarction who are younger than 45 years old.^{14,17}

Deficiencies in PAI-1 may lead to bleeding disorders due to excessive clot lysis. Deficiencies of α_2 -antiplasmin have been reported in four families to date and, in contrast, are associated with a severe hemorrhagic tendency which mimics congenital hemophilia. Deficiencies of TAFI have not been reported to be associated with a hemorrhagic diathesis, but elevated TAFI levels are felt to increase the risk for recurrent venous thromboembolism.¹⁸ Acquired abnormalities of the fibrinolytic system are much more common and are discussed in the next section on disseminated intravascular coagulation and related disorders.

An integrated system of serine proteases responds once the coagulation process is initiated in response to disruption of blood vessel integrity (Fig. 28-2). This response is balanced so that the same reaction that initiated thrombin formation and fibrin deposition also initiates a series of reactions to lyse the clot. Factor XIIa, with other components of the contact phase

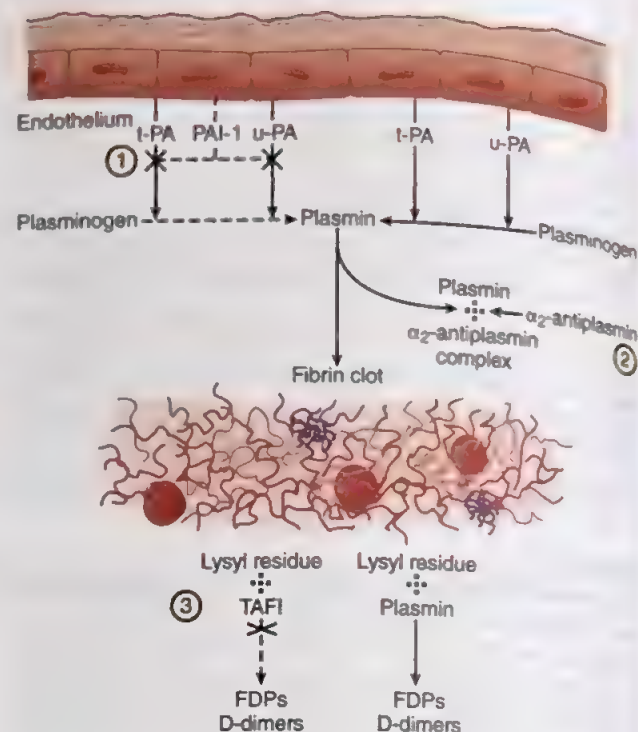


FIGURE 28-2 Control of fibrinolysis. Plasminogen activators tissue plasminogen activator (tPA) and, to a lesser degree, urokinase (u-PA) are synthesized in the endothelium. Both tPA and u-PA are rapidly inhibited by activated plasminogen activator inhibitor-1 (PAI-1), also synthesized in the endothelium. The interaction of tPA with PAI-1 regulates fibrinolysis, and under basal conditions, most tPA released is bound to PAI-1. Plasminogen-tPA binds to fibrin in clots generating plasmin at the LYSYL (lysine) residue site and clot lysis occurs resulting in the formation of fibrin degradation products (FDPs) including D-dimers. The plasmin inhibitor α_2 -antiplasmin binds to free circulating plasmin at the same lysine binding site, thus limiting plasmin activity to the area of fibrin deposition. Excess levels of thrombin activate the plasma protein thrombin activatable fibrinolysis inhibitor (TAFI), which binds with fibrin at the plasminogen binding site, thus preventing plasmin formation and fibrinolysis.

of coagulation, converts plasminogen to plasmin; thrombin stimulates endothelial cells to release tPA with subsequent plasmin generation. Excess tPA activity is controlled by the presence of plasminogen activator inhibitor. Because of the high affinity of plasmin for fibrin, most of these fibrinolytic processes take place at the site of fibrin deposition within the damaged blood vessel. The presence of plasmin inhibitors further ensures that the proteolytic process is limited to this area.

Disseminated Intravascular Coagulation

Under normal physiological conditions, damage to the vascular endothelium causes platelets to adhere to the site of injury, undergo activation, and aggregate. This reaction provides a rich phospholipid environment in which coagulation can proceed. Under most circumstances, the initial coagulant response involves endothelial cell tissue factor (TF) complexing with factor VII or VIIa to form TF-VIIa. This complex then rapidly converts factor X to Xa and factor IX to IXa. The TF-VIIa complex is short lived because of quick inhibition by the naturally occurring inhibitor, tissue

factor pathway inhibitor (TFPI). The latter forms an inactive quaternary complex of TF-VIIa-Xa-TFPI. Further coagulant response then depends on the formation of IXa-VIIIa through the activation of the intrinsic pathway to convert factor X to Xa resulting in a sustained burst of thrombin generation. Once thrombin is generated, fibrinogen is converted to fibrin, factor XIII to its activated form to make the clot insoluble, and thrombin limits coagulant activity by converting protein C to its activated form. The latter proteolytically cleaves any factor Va and VIIIa present, resulting in a reduction of further coagulant response. Endothelial cells are stimulated by thrombin to release tPA and single-chain urokinase to initiate the fibrinolytic process. Excess tPA and

single-chain urokinase are rapidly inhibited by PAI-1, and excess plasmin is bound to α_2 -antiplasmin (Fig. 28-3). Thus, further hemorrhage is prevented, and vascular repair is initiated to restore normal homeostasis.¹⁹

When damage occurs to a blood vessel, an integrated series of reactions involving the coagulation, fibrinolytic, kinin, and complement systems occurs on endothelial cells and platelets. The initial formation of the fibrin clot prevents further hemorrhage and initiates vascular repair. The subsequent clot lysis serves to reestablish blood flow and vascular integrity. This process is normally self-limited and localized. Under certain pathological stimuli, however, the coagulation response can be accentuated and overwhelm the normal inhibitory

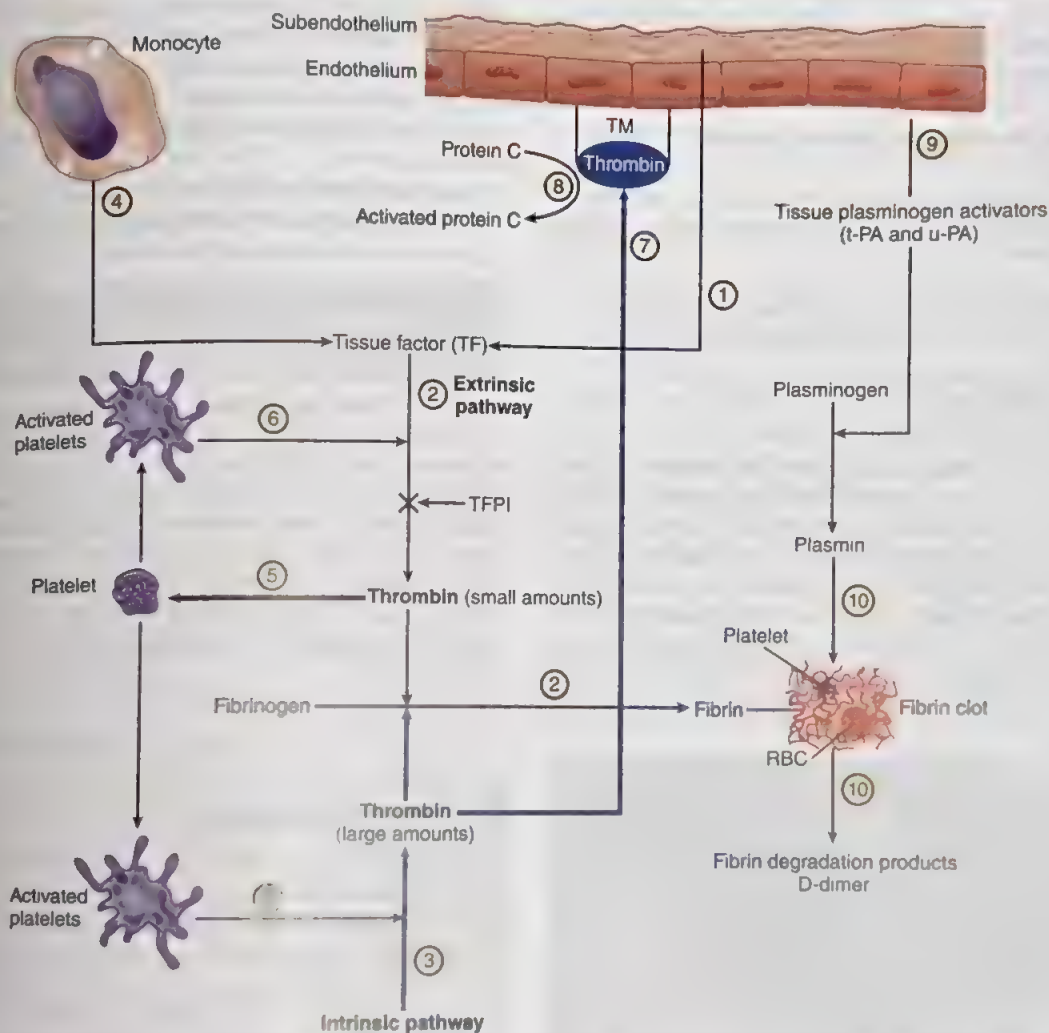


FIGURE 28-3 Integrated system of hemostasis. 1. Disruption of endothelial continuity releases tissue factor (TF). 2. In the presence of factor VII or VIIa, TF forms a complex with factor VII or VIIa leading to the conversion of factor X to Xa and rapid generation of small amounts of thrombin and fibrinogen to fibrin via the extrinsic pathway. In addition, the TF-factor VIIa-Xa complex is rapidly inhibited by the presence of tissue factor pathway inhibitor (TFPI). 3. The VIIa-TF complex also activates factor IX to IXa, initiating the intrinsic pathway and the formation of larger amounts of thrombin on the surface of activated platelets. 4. Enhanced expression of TF by monocytes also occurs in response to endotoxin or cytokines secondary to sepsis. 5. Thrombin stimulates platelet activation and the release of platelet agonists. 6. Activated platelets undergo shape change, resulting in the exposure of phospholipids and other factors V and VIII on the surface of the platelet membrane. 7. Secondly, and simultaneously, thrombin complexes with thrombomodulin (TM) on the endothelial surface. 8. Protein C, once bound to this complex, is rapidly converted to its activated state. 9. Activated protein C indirectly causes release of tissue plasminogen activator in cells from the subendothelium, in addition to direct stimulation and release of this glycoprotein by thrombin. 10. Plasmin-induced proteolysis of the fibrin clot results in the formation of fibrin degradation products.

mechanisms. Activation of the coagulation system under these circumstances causes consumption of the coagulation factors and platelets, with subsequent thrombus formation not only at the site of endothelial damage but in a random manner throughout the microcirculation.²⁰ This hemorrhagic syndrome is referred to as disseminated intravascular coagulation (DIC), defibrination syndrome, or consumptive coagulopathy.

Upon activation of the coagulation cascade, the fibrinolytic system is initiated. Regardless of the nature of the inciting stimulus, the pathophysiologic effect of this process reflects the balance between fibrin deposition (action of thrombin) and fibrinolysis (action of plasmin). Clinical manifestations can include diffuse hemorrhage (Fig. 28-4), due to depletion of platelets and coagulation factors, multiorgan dysfunction caused by vascular occlusion, or the occurrence of both conditions simultaneously in different areas of the microvasculature.

Triggering Mechanisms and Associated Clinical Disorders

The diverse stimuli capable of triggering the coagulation cascade in this manner all act through one or more of three mechanisms:²¹

1. Activation of the extrinsic coagulation pathway by the release of tissue factor or tissue thromboplastin-like substance
2. Direct activation of factors X or II
3. Activation of the intrinsic pathway

Tissue factor release/expression is the primary initiator in most cases of DIC, either due to tissue thromboplastin entry into the circulation, extensive injury to the vascular endothelium exposing tissue factor in the subendothelium, or enhanced expression of tissue factor by monocytes in response to endotoxin or cytokines (tissue necrosis factor [TNF], IL-1, and IL-6) secondary to sepsis involving both gram-negative and gram-positive organisms or inflammation. In certain conditions, specifically obstetrical complications

or cranial injuries, thromboplastin-like substances released into the circulation. These substances have stimulatory properties similar to tissue factor and activate the extrinsic pathway, leading to DIC. Macrophage stimulation results in increased TNF being released, with subsequent downregulation of thrombomodulin sites on the endothelial cells and impairment of fibrinolysis. The intrinsic system involvement is usually indirect and occurs secondary to activation of the coagulation process by initiators of the extrinsic system. DIC caused by direct activation of factor VII, as seen after massive injury or in certain obstetrical complications, results from release of tissue thromboplastin from the injured tissue or from amniotic fluid entering the circulation. Certain tumors, particularly mucinous adenocarcinomas, are rich in thromboplastin-like material and may act through the same mechanism. The coagulopathy seen with red blood cell lysis after mismatched blood transfusions may be caused by the release of thromboplastin-like activity from the stroma of these cells; however, evidence suggests that immune complex formation may be the prime initiator of the coagulopathy response.

Direct activation of coagulation factors X and II can also occur in the presence of proteolytic enzymes. The venom of certain snakes act through this mechanism (e.g., Russell's viper venom activates factor X, whereas venom from the sand rattlesnake causes direct conversion of prothrombin to thrombin). Certain malignancies have also been reported to have TF or direct factor X activation by a calcium-dependent cysteine protease, and these properties may account for the DIC seen in these states.²² Although the hemorrhagic disorder noted in acute promyelocytic leukemia (APL) is listed as secondary to DIC, evidence suggests that the bleeding diathesis seen in these patients may also involve primary fibrinolysis in addition to DIC. Leukemic promyelocytes are rich in annexin II, a surface phospholipid, which can bind tPA and plasminogen. Conversion of the plasminogen to plasmin then initiates the fibrinolytic process without thrombin generation.²³



FIGURE 28-4 Diffuse hemorrhage, a clinical manifestation in a patient with disseminated intravascular coagulation (DIC). Note the multiple cutaneous ecchymoses.

ADVANCED CONTENT

Although activation of the intrinsic system can result in DIC, this is an uncommon mechanism. The exact sequence of intermediary events by which certain stimuli initiate coagulation is well understood, but with other stimuli this process remains uncertain. All pathological stimuli that result in activation of the intrinsic system most likely do so indirectly by means of first inducing endothelial cell damage with subsequent exposure of the subendothelium. Platelet adherence and aggregation and factor XII activation can then occur. This is the proposed mechanism of DIC associated with anoxia and immune complex formation, but activation through the extrinsic system may also play a role. New insight regarding sepsis now suggests

that the mechanism of DIC induced by endotoxins, the lipopolysaccharide constituents of gram-negative organisms, may be more complicated, with endotoxin inducing release of a number of cytokines, including tumor necrosis factor- α (TNF α and IL-1), and the primary activation of coagulation occurring through the extrinsic (tissue factor-dependent) pathway rather than the intrinsic pathway.²⁴ TNF- α is capable of inducing tissue factor activity in monocytes on both the luminal and subendothelial surfaces of endothelial cells. Tissue factor can bind and activate factor VII; the complex of TF-VIIa can then activate factor X, with subsequent thrombin formation. Binding of granulocytes to the endothelial receptors (selectins) induces the release of granulocyte cathepsins and elastases, which then lead to organ damage. The intrinsic system of coagulation participates in the DIC process by activating the kinin system through the interaction of factor XIIa and prekallikrein, with high molecular weight kininogen converted to kinins. Kinins cause decreased vascular tone, increased vascular permeability, hypotension, and, if severe, lead to shock.²⁵ A list of clinical disorders associated with these triggering mechanisms is shown in Box 28-1.

BOX 28-1 Clinical Conditions Associated With Disseminated Intravascular Coagulation

Thromboplastin Release—Factor VII Activation

- Placental abruption
- Trauma
- Fat emboli syndrome
- Mucin-secreting adenocarcinoma
- Sepsis*
- Promyelocytic leukemia
- Retained dead fetus syndrome
- Acute intravascular hemolysis*
- Amniotic fluid embolus*
- Cardiopulmonary bypass surgery

Endothelial Cell Damage—TF Release

- Immune complex disease
- Intravascular hemolysis*
- Liver disease*
- Heat stroke
- Sepsis*
- Burns
- Vasculitis
- Anoxia
- Acidosis

Factor X/II Activation

- Snake venoms
- Acute pancreatitis
- Liver disease*
- Fat emboli syndrome*

*More than one mechanism may be involved.

CRITICAL THINKING QUESTION

28-2 What characteristics of DIC would make it difficult to detect through routine coagulation studies?

Clinical Presentation

To a great extent, the clinical presentation of DIC depends on which of the proteolytic processes (coagulant or fibrinolytic) is dominant. This allows for a wide spectrum ranging from an acute, severe hemorrhagic disorder (acute DIC or uncompensated DIC) to a low-grade disorder with predominantly thrombotic manifestations or laboratory abnormalities only (chronic DIC or compensated DIC) without clinical evidence of hemorrhage or thrombosis.

In acute DIC, bleeding manifestations of almost every kind have been described. Ecchymosis, petechiae, and bleeding from previously intact venipuncture sites or indwelling catheters have been observed in many patients.

Purpura fulminans is a rare, life-threatening condition that can occur in acute DIC, causing extensive tissue thrombosis and hemorrhagic skin necrosis. It is characterized by symmetric ecchymosis of the lower extremities and buttocks. These ecchymotic lesions can become necrotic. Purpura fulminans may develop in patients with DIC due to an acquired protein C deficiency from consumption.²⁶

In chronic DIC, a small amount of activating stimulus is released intermittently, resulting in subtle hemostatic dysfunction. However, destruction and production of coagulation factors is balanced with a potential increase in thrombotic risk without obvious clinical symptoms.

A number of factors are important in determining the final clinical picture, including the magnitude and duration of the triggering stimulus; the functional ability of reticuloendothelial system, particularly the liver, to remove from circulation activated coagulation factors, fibrin monomers, fibrin/fibrinogen products, as well as immune complexes; the compensatory ability of the liver and the bone marrow to accelerate clotting factor and platelet production; and, finally, the extent to which any particular organ is involved with hemorrhage or thrombus.²⁷

Laboratory Diagnosis

To confirm a laboratory diagnosis of DIC, evidence of activation of procoagulant and fibrinolytic components, inhibitor consumption, and end organ failure should be confirmed. The laboratory findings in patients with DIC reflect the direct or indirect effects of excess thrombin and plasmin generation (Table 28-2). The constellation of laboratory abnormalities in any particular patient depends on the nature, magnitude, and duration of the triggering stimulus; the compensatory capacity available; and the underlying disease state. No single test is diagnostic of DIC; however, in the appropriate clinical setting (patient history and the type of bleeding and/or thrombosis), a battery of tests can ensure a diagnosis of DIC.

Examination of a blood smear or tissue biopsy may reveal evidence of acute bleeding, accelerated red blood cell

TABLE 28-2 Laboratory Tests to Detect Excess Thrombin and/or Plasmin Activity

Excess Protease	Effect	Laboratory Tests
Thrombin	• Fibrinogen utilization	• Fibrinogen concentration, thrombin/reptilase time, prothrombin and activated partial thromboplastin times
	• Utilization/degradation of other coagulation factors	• Prothrombin and activated partial thromboplastin times
	• Fibrin monomer generation	• Soluble fibrin monomer complexes
	• Fibrinopeptide A/B release	• Fibrinopeptides A and B
	• Platelet aggregation/release	• Platelet count, β -thromboglobulin, and platelet factor 4
	• Antithrombin complex formation	• Thrombin-antithrombin complexes, Antithrombin level
Thrombin/plasmin	• Proteolysis of fibrinogen/fibrin	• Fibrin degradation products, D-dimer concentration, B- β 15-42 peptide assay
	• Release/activate	• Tissue plasminogen
	• Plasminogen activators and inhibitors	• Activator level, plasminogen activator inhibitor level
	• Soluble fibrin monomer/fibrinogen FDP complexes	• Soluble fibrin monomer complexes
Plasmin	• Plasminogen utilization	• Plasminogen concentration
	• Proteolysis of fibrinogen/fibrin	• FDPs, thrombin/reptilase times, platelet aggregation and release tests
	• Complexes with inhibitor	• α_2 -Plasmin, inhibitor concentration, α_2 -plasmin inhibitor-plasmin complexes
	• Proteolysis of factors V/VIII	• Factor assays

destruction, or signs of underlying disease. If the fibrin deposition does not completely occlude the lumen of the damaged blood vessel, red blood cells may undergo a shearing effect as they traverse this area, with resultant fragmentation and the development of a microangiopathic hemolytic anemia (Fig. 28-5) or the lumen may be completely occluded by the thrombus, negating the formation of fragmented RBCs in the circulation (Fig. 28-6). Microangiopathic hemolytic anemia with schistocytes may be evident on the blood smear in approximately 50% of patients with acute DIC.²⁸

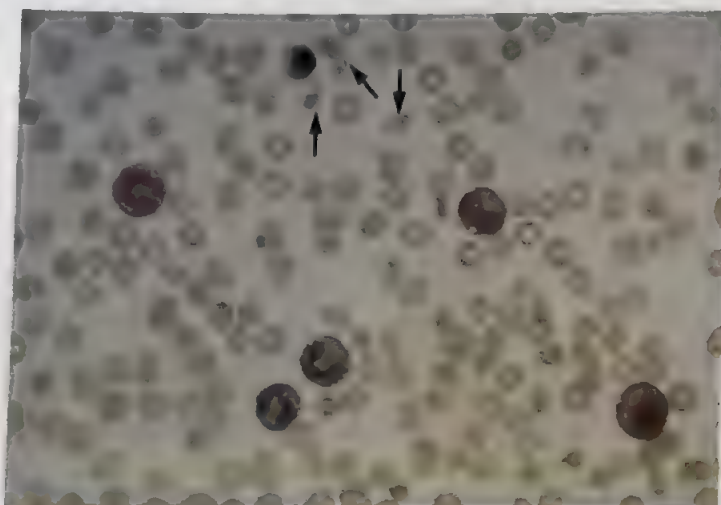


FIGURE 28-5 DIC (peripheral blood). Note presence of schistocytes (arrows) and nucleated red blood cells (top border).

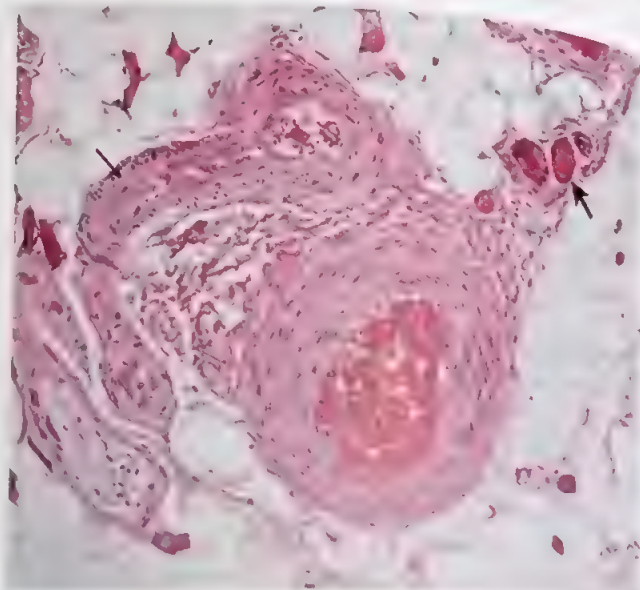


FIGURE 28-6 DIC (skin biopsy). Note both partial (small arrow) and complete (large arrow) occlusion of blood vessels by RBC/fibrin clot.

Thrombocytopenia is an early and consistent sign of acute DIC. Platelets are consumed during the coagulation process and their contents released, resulting in thrombocytopenia and elevated plasma levels of the platelet-specific proteins, thromboglobulin, and platelet factor 4. The platelet count is most often mildly or moderately reduced; platelet counts under 20,000 / μ L are not commonly seen.

Basic coagulation tests should be performed first when making the diagnosis of DIC. These tests include the prothrombin time (PT), activated partial thromboplastin time (aPTT or APTT), fibrinogen levels, FDP or D-dimer, and thrombin time. The PT, aPTT, and thrombin time are prolonged in most patients with acute DIC due to utilization and degradation of the clotting factors. Early in the course of the disease or with chronic DIC, the aPTT may be normal due to elevated factor VIII levels. Hypofibrinogenemia is more common with acute rather than chronic DIC. Fibrinogen levels are most consistently affected by DIC, but plasma fibrinogen may be only slightly decreased or normal because it is an acute phase reactant protein.²⁹

Tests for the secondary activation of the fibrinolytic system in DIC are primarily directed at demonstrating the action of plasmin on fibrin/fibrinogen. As has already been mentioned, a series of cleavage products are formed, the fibrin/fibrinogen degradation products. The anticoagulant action of these fragments has been noted in the previous section. Several immunological tests are available to measure one or more of the fibrinogen fragments and can yield quantitative information on the degree of fibrinolysis. Because of its ease of performance and specificity (i.e., evidence of plasmin action on cross-linked fibrin, indicating coagulation has occurred), the D-dimer latex microparticle agglutination assays are the immunological assays of choice, and automated techniques are available to quantitate levels.

Plasma levels of coagulation natural inhibitors, such as antithrombin or protein C, are decreased in approximately 90% of acute DIC patients. During severe inflammatory responses, antithrombin levels are decreased due to consumption and impaired synthesis. Antithrombin complexes with thrombin and activated factor X, resulting in diminished plasma levels of antithrombin. The presence of thrombin-antithrombin complexes can be determined using an enzyme-antithrombin complex assay (ELISA) system. Protein C deficiency is due to enhanced consumption and impaired liver synthesis. Decreased levels of protein C are seen in patients with meningococcal septicemia and may play an important role in the formation purpura fulminans.³⁰

CRITICAL THINKING QUESTION

28-3 Why do some patients with DIC present with schistocytes on peripheral blood smear?

ADVANCED CONTENT

Conversion of prothrombin to thrombin is the result of cleavage from the parent molecule of a small, inactive peptide referred to as *prothrombin fragment F1.2*. Measurement of this activation peptide has been utilized primarily for diagnosis of a hypercoagulable state; however, it may have equal utility as a molecular marker for DIC.

Specific tests for direct evidence of thrombin activity relate to the action of thrombin on fibrinogen. Other than

certain snake venoms, thrombin is the only enzyme that releases the specific peptides fibrinopeptide A and B from the fibrinogen molecule. Both ELISA and radioimmunoassays are available commercially to measure each fibrinopeptide. The major drawback to measuring these peptides, paradoxically, is the extreme sensitivity of the assay. Elevated levels can be seen in clinical conditions in which thrombin is only transiently generated. As a consequence of fibrinopeptide release, soluble fibrin monomers are formed that are capable of forming complexes with intact fibrinogen molecules or with fibrin/fibrinogen degradation products. Soluble fibrin monomer complex formation can be measured using a commercially available qualitative hemagglutination assay of human erythrocytes coated with fibrin monomers. Positive test results are indirect indications that thrombin was generated and the coagulation system activated. Direct measurement of the plasminogen concentration in plasma can also be performed, and commercial assays are available.

An indication of increased plasminogen activator activity seen in early stages of DIC can be obtained by performing a euglobulin lysis time. The euglobulin fraction of plasma contains plasminogen, plasminogen activator, plasmin, and fibrinogen. The rapidity of lysis of the fibrin clot is directly related to plasminogen activator levels. The sensitivity of this global assay is limited. As discussed, direct quantitation of each component using commercially available kits has replaced the euglobulin lysis time. Assays for α_2 -antiplasmin inhibitor levels and for circulating plasmin- α_2 -antiplasmin inhibitor complexes are available for clinical use.³¹

Table 28-3 lists available laboratory tests used to diagnosis DIC with their associated results, depending on the patient's level of compensation. Three generalized clinical states of DIC are described in this table along with the typical laboratory abnormalities associated with each.

The **acute (decompensated) DIC** state refers to a condition in which active hemorrhage is evident and the consumption of the coagulation factors and platelets exceeds the capacity to increase the synthesis of these components. In the **chronic (compensated) state**, laboratory evidence of an accelerated coagulation and fibrinolytic process is evident (increased FPA, soluble fibrin monomer complexes, increased β -thromboglobulin, increased FDPs or D-dimer levels, presence of plasmin- α_2 -antiplasmin complexes), but the rate of synthesis of the coagulation components is balanced with the rate of destruction. Because of this balance, the PT, aPTT, thrombin time, and platelet count are usually normal or only mildly abnormal. Confirmation of DIC is based on finding evidence of coagulation activation peptides (FPA, prothrombin fragment 1.2) and complexes of activated coagulant/fibrinolytic components (thrombin-antithrombin, plasmin- α_2 -antiplasmin) in addition to increased fibrin(ogen) degradation products and elevated β -thromboglobulin or platelet factor 4 (PF4) levels.

TABLE 28-3 Laboratory Tests to Diagnose DIC

Test	Acute (Decompensated)	Chronic (Compensated)
Routine Tests		
Prothrombin time	I	N
Activated partial thromboplastin	I	N/D
Thrombin time/reptilase time	I	N/I
Fibrinogen	D	N
Platelet count	D	N/D
D-Dimer/fibrin(ogen) degradation products	I	N/I
Antithrombin	D	N/D
Special Tests		
Coagulation factor levels	D	N/D
Fibrinopeptide A	I	I
Plasminogen	D	N/D
Plasmin- α_2 -plasmin	I	N/I
β -Thromboglobulin/platelet factor 4 levels	I	I
Thrombin-antithrombin complexes	I	I
Prothrombin fragment F1.2	I	I
Euglobulin lysis test	N/I/D	N/D
Soluble fibrin monomer complexes	P	P/Ng

I = increased; D = decreased; N = normal; P = positive; Ng = negative.

The hypercoagulable state is the result of excess thrombin present in the plasma, with a delayed or lessened plasmin response. In this condition, evidence of coagulation activation is apparent (increased levels of FPA, thrombin-antithrombin complexes, prothrombin fragment 1.2, α -thromboglobulin), but all fibrinolytic activation markers are absent or minimally increased. A characteristic finding in this form of DIC is a shortened aPTT. These clinical states are not static, and it is not unusual for one to evolve into one of the others, depending on the nature of the underlying disease process and response to therapy.

Treatment

Treatment of DIC is essentially two-fold: treatment or removal of the underlying pathological stimulus and maintenance of blood volume and hemostatic function. Dramatic improvement in the patient's clinical status with abrupt cessation of bleeding and normalization of the coagulation abnormalities can be seen in certain cases of DIC with removal of the underlying pathological stimulus alone (e.g., DIC associated

with retained dead fetus). In cases of DIC associated with septicemia, appropriate antibiotic therapy is important to control the pathological process (i.e., bacterial or endotoxin-induced vascular damage).

Blood component replacement therapy with transfusion of packed red blood cells, fresh frozen plasma, and platelets to maintain blood volume and support hemostatic function is indicated in patients with active bleeding or those whose compensatory capacity is limited. In addition to fresh plasma, cryoprecipitate (enriched in fibrinogen, factor VIII, and fibronectin) and prothrombin complex (enriched in vitamin K-dependent clotting factors) are often used as supplemental sources of blood component therapy.

The administration of unfractionated (UF) heparin and low molecular weight heparin (LMWH) is a therapeutic option in DIC. This therapy option is based on the premise that the underlying pathological cause of DIC is generation of excess thrombin, leading to thrombosis, especially of small vessels. This process has the greatest effect on morbidity and mortality. UF or LMWH should theoretically slow or stop the coagulation process by complexing with antithrombin to inhibit thrombin or factor Xa. Its use can result in increased bleeding, and because heparin itself affects a number of coagulation tests, it is often difficult to monitor the effect of coagulation therapy. Heparin should be utilized and is most effective in cases of sepsis-related DIC. Heparin therapy has also shown to be effective in patients that develop purpura fulminans.³⁹

Recombinant activated protein C is an additional therapeutic agent used in this group of patients because the inflammatory response to sepsis-induced DIC with multiorgan failure. A reduced mortality and improvement in organ function compared with control subjects have been reported.³⁷ Other treatments focus on restoring deficiencies of natural coagulation pathway inhibitors, such as antithrombin and protein C, especially when there is documentation of deficiencies of these proteins.³⁹

When major peripheral vessels are occluded as part of the hypercoagulable process, the use of fibrinolytic agents (recombinant tPA, streptokinase, or urokinase) may be indicated as an initial management choice with subsequent heparinization, but clinical experience with this form of therapy is minimal.

CRITICAL THINKING QUESTION

28-4 What is the main goal of treatment for DIC?

Related Disorders

Primary fibrinolysis is an unusual situation in which plasmin is formed in the absence of activation of the coagulation cascade. The clinical presentation in this disorder is similar to that in DIC, with diffuse hemorrhage occurring as a result of increased plasma fibrinolytic activity. Several mechanisms can initiate this process. The presence of proteolytic enzymes

in plasma that are capable of either directly or indirectly converting plasminogen into plasmin can occur in certain disease states. The genitourinary system is enriched in urokinases, which can enter the systemic circulation after various urological procedures. The fibrinolytic state seen with metastatic carcinoma is another example of this mechanism.

The basis for the hemorrhagic state seen after cardiopulmonary bypass surgery is complex, with platelet dysfunction and hemodilution of the plasminogen-plasmin system with increased fibrinolytic activity is also well documented.

The failure of the hepatic clearance mechanism to remove plasminogen activator accounts for the increased fibrinolytic activity seen in a variety of hepatic disorders, particularly cirrhosis. Under normal circumstances, the hepatic reticuloendothelial system removes not only activated clotting proteins but also plasminogen activator from the systemic circulation. When this function is impaired because of hepatic dysfunction, as in patients who have portocaval shunting procedures, the removal of plasminogen activator is less than adequate and hyperplasminemia occurs, with resultant hemorrhage. Increased fibrinolytic activity can also be seen in patients undergoing liver transplantation, especially during the reperfusion phase after reanastomosis of vessels.

The occurrence of DIC with secondary fibrinolysis is well documented in patients with acute promyelocytic leukemia, although as previously discussed, this disorder clearly causes primary fibrinolysis through surface membrane annexin II binding of tPA and plasminogen. It is now recognized, however, that the coagulopathy these patients develop may also result from a primary fibrinolysis. The mechanism(s) is unsettled but may involve direct activation by the leukemic cells with release of a urokinase-type or tissue-type plasminogen activator.³³

The coagulation abnormalities seen in these fibrinolytic disorders are similar to those in DIC, with prolonged PT, APTT, and thrombin times. These defects result from the hypofibrinogenemic state induced by the proteolytic cleavage of fibrinogen by excess plasmin, in addition to the catabolic effect of this enzyme on factors V and VIII. FDP concentrations are increased and, as previously noted, will further interfere with coagulation by acting as antithrombins and inhibitors of fibrin polymerization. With the excess plasmin activity, the euglobulin lysis time is typically shortened. Because thrombin is not generated during this pathological process, laboratory findings that are the direct result of thrombin activity will be absent. Several laboratory tests can serve to readily distinguish primary fibrinolysis from DIC. In primary fibrinolysis, the platelet count is typically normal; fibrinogen levels and fibrinogen A and B levels are not elevated, and circulating fibrin-monomer complexes and elevated D-dimer levels are absent in contrast to the results in DIC (Table 28-4).

TABLE 28-4 Laboratory Differentiation Between DIC and Primary Fibrinolysis

Laboratory Test	DIC	Primary Fibrinolysis
Prothrombin time	I	I
Activated partial thromboplastin time	I	I
Thrombin time	I	I
Fibrinogen	D	D
Platelet count	D	N
Fibrinogen A	I	N
SFMC	I	N
TAT	I	N
FDPs	I	I
D-Dimers	I	N

SFMC = soluble fibrin monomer complexes; TAT = thrombin-antithrombin complexes; FDPs = fibrin/fibrinogen degradation products.

Thrombotic thrombocytopenic purpura is the syndrome in which fibrin and platelet thrombi are formed diffusely throughout the microvasculature, in contrast to the localized thrombus formation seen in DIC. The clinical picture consists of a pentad of findings:

1. Fever
2. Microangiopathic hemolytic anemia
3. Thrombocytopenia
4. Azotemia
5. Vacillating neurological deficits

Despite fibrin and platelet deposition, this disorder is not typically associated with excessive activation of the coagulation system. The pathological mechanism in this disorder is either a hereditary/familial deficiency of von Willebrand cleaving enzyme (ADAMTS-13) in endothelial cells or the development of an acquired IgG inhibitory antibody to the enzyme that results in the secretion and circulation of ultralarge high molecular weight von Willebrand factor multimers that spontaneously bind to circulating platelets and form occlusive platelet thrombi in the vasculature.³⁴ An abnormality of the fibrinolytic system can also be present in some patients with diminished or absent fibrinolytic activity, particularly of tPA, in plasma and blood vessels affected with microthrombi. It is likely these are secondary changes. Therapy has not been standardized, but antiplatelet drugs (e.g., aspirin or dipyridamole), plasmapheresis, and exchange transfusion have been used either singularly or in combination with variable success.

SUMMARY CHART

- Normal hemostasis is the result of the balanced interaction of the vascular endothelium and platelets with four biochemical systems: coagulation, fibrinolytic, kinin, and complement systems
- The molecular components of the fibrinolytic system consist of plasminogen, plasmin, plasminogen activators, PAI-1, plasmin inhibitors, thrombomodulin, thrombin-activatable fibrinolysis inhibitor, and fibrin(ogen).
- Native plasminogen is a single-chain plasma zymogen of approximately 90 kD that circulates in a lysine and glutamic acid form.
- Plasminogen activators include tissue plasminogen activator (tPA), urokinase, and streptokinase.
- The primary inhibitor of plasminogen activators is PAI-1
- Thrombin complexed with soluble and cell-bound thrombomodulin activates procarboxypeptidase B, which binds to the plasminogen binding site on fibrin, preventing plasmin formation; this is known as thrombin-activatable fibrinolysis inhibitor.
- Plasmin is a serine protease that has the ability to degrade fibrin in clots and native fibrinogen in circulation into a series of well-characterized end products known as fibrin/fibrinogen degradation products (FDPs).
- The primary physiological inhibitor of plasmin *in vivo* is α_2 -antiplasmin; it binds to the lysine binding site on plasmin in a 1:1 molar ratio in an irreversible manner.
- Disseminated intravascular coagulation (DIC) occurs when the coagulation response has been accentuated and the normal inhibitory mechanisms are overwhelmed and cannot stop thrombus formation. Coagulation factors and platelets are consumed with subsequent thrombus formation throughout the microcirculation.
- Triggering mechanisms of DIC may include activation of the extrinsic coagulation pathway by release of tissue thromboplastin, direct activation of factor X or II by cysteine protease, and occasionally activation of the intrinsic system by thromboplastins
- Acute DIC (decompensated) state refers to a condition in which active hemorrhage is evident and consumption of the coagulation factors and platelets exceeds the capacity to increase the synthesis of these components
- Chronic DIC (compensated) state refers to laboratory evidence of accelerated coagulation and fibrinolysis, with the rate of synthesis of the coagulation components balanced with the rate of destruction.
- The hypercoagulable state of DIC is the result of excess thrombin present in the plasma, with a delayed or weakened plasmin response.
- Therapy for DIC is aimed at treatment or removal of the underlying pathological stimulus and maintenance of blood volume and hemostatic function.
- Primary fibrinolysis is a condition in which plasmin is formed in the absence of coagulation processes; prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time, and FDP are increased, and euglobulin lysis time is shortened.
- The FDP fragment X is capable of clotting and exerts an anticoagulant effect by competing with fibrinogen for thrombin; fragments D and Y are unable to form a clot. D-Dimer represents a specific proteolytic product of fibrin degradation by plasmin.
- Clinical conditions associated with DIC may include placental abruption, trauma, sepsis, promyelocytic leukemia, cardiopulmonary bypass, immune complex disease, burns, anoxia, liver disease, and snake bites.

CASE STUDY 28-1

A 32-year-old woman in her 36th week of gestation noted the sudden onset of lower abdominal pain and profuse vaginal bleeding. She was rushed to the emergency department. On examination, she was noted to be hypotensive, with a blood pressure of 70/40 and marked tachycardia. Large ecchymoses and continuous oozing of blood from venipuncture sites were evident. A fetal heart tone was barely audible. Births of her other children were uncomplicated, and the family history was negative for a hemorrhagic diathesis.

Initial coagulation studies revealed PT of 26 seconds (normal is 11 to 13 seconds); aPTT of 84 seconds (normal is 24 to 30 seconds); platelet count of 20,000/ μ L (normal is 150,000 to 400,000/ μ L); fibrinogen 88 mg/dL (normal is 145 to 350 mg/dL); FDPs, greater than 40 μ g/mL (normal is less than 10 μ g/mL). A blood smear showed numerous red blood cell fragments present.

A diagnosis of DIC was made based on the patient's clinical presentation and the supportive laboratory data. The patient was placed on intravenous fluids to maintain her

CASE STUDY 28-1—cont'd

blood pressure and given 2 units of fresh frozen plasma and 10 units of platelets. She was taken to the operating room and underwent a caesarean section. Her bleeding abated postoperatively, and all coagulation parameters returned to normal within 36 hours.

This case is illustrative of an obstetric complication, placental abruption, which resulted in an acute DIC syndrome. Several triggering mechanisms have been postulated as an explanation for the underlying coagulopathy in this disorder, including the release of thromboplastin-like material from the amniotic fluid and tissue necrosis in the area of the retroperitoneal hemorrhage. The laboratory parameters are consistent with a consumptive coagulopathy and secondary fibrinolysis. With the delivery and removal of the placenta, the source of the triggering mechanism was removed, and the pathological process stopped. Restoration of normal hemostatic parameters occurs within hours postoperatively and usually no further blood component replacement therapy is required.

QUESTIONS

1. Which lab tests indicate that the patient is experiencing bleeding issues and thrombosis?
2. Does this scenario align with a diagnosis of DIC? If so, is it acute or chronic?
3. Why did the C-section help this patient?

ANSWERS

1. Prolonged PT and aPTT indicate the patient is bleeding. Low platelets and fibrinogen indicate the consumption of factors. FDPs indicate thrombosis. Red blood cell fragments indicate that shearing is taking place within the blood vessel lumen, indicative of clotting.
2. Yes, DIC involves both bleeding and clotting issues. The levels of hemorrhage and thrombosis seen in this patient indicate acute DIC is present.
3. Complications from pregnancy are a trigger for DIC and removal of the underlying trigger is the best treatment.

REVIEW QUESTIONS

1. D-Dimer formation is the result of the action of plasmin on:
 - a. Fibrin monomer
 - b. Fibrinogen
 - c. Cross-linked fibrin
 - d. FDPs
2. What causes inactivation of factors Va and VIIIa?
 - a. Thrombin
 - b. Protein S
 - c. tPA
 - d. Activated protein C
3. In primary fibrinolysis, which of the following laboratory tests will be abnormal?
 - a. Platelet count
 - b. D-Dimer level
 - c. Fibrinopeptide A level
 - d. Thrombin time
4. In DIC presenting clinically as a hypercoagulable state, it is not unusual for which of the following coagulation times to be paradoxically shortened?
 - a. Reptilase time
 - b. Euglobulin lysis time
 - c. aPTT
 - d. Thrombin time
5. What is the primary inhibitor of the fibrinolytic system?
 - a. Antithrombin
 - b. α_2 -Antiplasmin
 - c. Protein C
 - d. α_2 -Macroglobulin
6. What is the primary tissue source for tissue plasminogen activator (tPA)?
 - a. Neutrophils
 - b. Mast cells
 - c. Endothelial cells
 - d. Pluripotential stem cells
7. Which of the following is an abnormal RBC morphology that can be seen in acute DIC?
 - a. Tear drop
 - b. Target cells
 - c. Schistocytes
 - d. Acanthocytes
8. What is the primary inhibitor of plasminogen activators?
 - a. PAI-1
 - b. PAI-2
 - c. TAFI
 - d. Thrombomodulin

REVIEW QUESTIONS—cont'd

9. Which is a characteristic of compensated DIC?
 - a. Severe hemorrhage
 - b. Evidence of increased fibrinolysis
 - c. Thrombocytopenia
 - d. Decreased antithrombin
10. Thrombin-activatable fibrinolysis inhibitor (TAFI) circulates in plasma as:
 - a. Plasminogen
 - b. Thrombomodulin
 - c. Fibrinogen
 - d. Procarboxypeptidase B
11. What is the main goal of the fibrinolytic system?
 - a. Formation of a stable fibrin clot
 - b. Lysis of the fibrin clot and reestablishment of blood flow
 - c. Activation of platelets
 - d. Activation of coagulation factors
12. Which of the following is a mechanism by which plasminogen is activated?
 - a. Endogenous activators
 - b. Phagocytosis
 - c. Intrinsic activators
 - d. Complement
13. Which of the following deficiencies results in a bleeding disorder?
 - a. Plasminogen
 - b. tPA
 - c. PAI-1
 - d. uPA
14. Which of the following is a trigger for DIC?
 - a. Direct activation of Factor VIII
 - b. Sepsis
 - c. Activation of complement
 - d. Direct activation of Factor XIII
15. How does chronic DIC present clinically?
 - a. Laboratory evidence only of thrombosis
 - b. Severe hemorrhagic disorder
 - c. Purpura fulminans
 - d. Necrotic lesions
16. What is the most effective treatment of DIC?
 - a. Attacking DIC symptoms directly
 - b. RBC transfusion without plasma transfusion
 - c. Attacking the underlying pathological stimulus
 - d. Factor transfusion

See answers at the back of this book.

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Introduction to Thrombosis and Anticoagulant Therapy

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CHAPTER OUTLINE

History	Factor XII Deficiency	Complete History and Physical Examination
Regulation of Coagulation and Fibrinolysis	Dysfibrinogenemia	Conditions That Can Interfere With Test Results
Role of Endothelium	Elevated Plasma Factor VIII Coagulant Activity	Testing in the Appropriate Clinical Setting
Platelets	Lipoprotein a and Thrombosis	Functional Assays
Procoagulant Factors and Thrombin Generation	Other Coagulant Factors Associated With Thrombosis	Testing Considerations
Natural Inhibitors of Coagulation Factors (Plasma Components)	Acquired Thrombotic Disorders	Anticoagulant Therapy
Fibrinolytic System	Lupus Anticoagulant/Antiphospholipid Syndrome	Unfractionated Heparin Therapy
Inherited Thrombophilia	Heparin-Induced Thrombocytopenia	Low Molecular Weight Heparin
Activated Protein C Resistance	Other Acquired Conditions Associated With Thrombosis	Vitamin K antagonists
Protein C Deficiency	Thrombosis With Pregnancy and Use of Oral Contraceptives	Direct Oral Anticoagulants (DOACs)
Protein S Deficiency	Thrombosis and Nephrotic Syndrome	Antiplatelet Agents
Antithrombin Deficiency	Thrombosis in Cancers and Other Conditions	Thrombolytic Therapy
Prothrombin (F2) G20210A Mutation	Diagnostic Approach and Issues in Laboratory Testing	Case Study 29-1
Hyperhomocysteinemia		Case Study 29-2
Tissue Factor Pathway Inhibitor Deficiency		Summary Chart
		Review Questions
		References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- | | |
|---|---|
| <p>29-1 Name the major factors that contribute to the risk of thrombosis.</p> <p>29-2 Identify the endothelial contributions to anticoagulation and thrombotic activity.</p> <p>29-3 Describe the activity of thrombin.</p> <p>29-4 Explain the role of antithrombin.</p> <p>29-5 Compare and contrast protein C (PC) and protein S (PS).</p> <p>29-6 Outline the inherited causes of thrombophilia based on demographic, clinical, and lab data.</p> <p>29-7 Explain why lupus anticoagulant testing is so complex.</p> | <p>29-8 Describe the mechanism for heparin-induced thrombocytopenia.</p> <p>29-9 Identify acquired causes of thrombophilia in various patient cases.</p> <p>29-10 Detail the purposes of testing for hypercoagulable states.</p> <p>29-11 List lab tests for evaluation of patients with hypercoagulable states.</p> <p>29-12 Name anticoagulant therapies used to treat hypercoagulable states.</p> <p>29-13 List standard lab testing for monitoring anticoagulant therapies.</p> |
|---|---|

History

More than 150 years ago, Virchow called attention to the fundamental processes involved in the pathogenesis of thrombosis: (1) the role of the blood vessel, (2) the flow of the blood within the vessel, and (3) the chemistry of the blood itself. Physicians and the public alike are well versed regarding risk factors for the arteriovascular disease of atherosclerosis that manifests primarily as stroke and myocardial infarction (MI). The risk factors include hypercholesterolemia, hypertension, and tobacco use. In these arterial vasculopathic disorders, thrombosis is often the final mechanism of occlusion, usually secondary to an atherosclerotic lesion. The lesions demonstrate Virchow's triad: a disrupted vessel lining, a turbulent flow of blood, and alterations in the concentrations of coagulation factors, which all contribute to the risk of thrombus formation in this setting.

Altered flow within the blood vessel is a well-recognized problem, particularly in the diseased vessel and in the immobilized or postoperative patient. The slowing of blood flow by prolonged sitting, as in long airplane or automobile trips, or the creation of turbulent flow present in atherosclerotic vessels can precipitate thrombosis, especially if added risk factors for thrombophilia exist. If large blood clots form in the veins of a patient, an unstable segment may break off and float to the lung vasculature. This potentially deadly pulmonary embolism (PE), among other complications, is the main reason for the widespread use of prophylactic anticoagulation medication within inpatient settings. Other conditions that promote a hypercoagulable state and promote indications for anticoagulation are chronic atrial fibrillation, artificial heart valves, and connection to bypass machines in patients undergoing cardiac surgery. Altered blood flow and some platelet activation mechanisms contribute to the hypercoagulable state in these conditions.

Until the mid-1980s, the laboratory had little to offer in the identification of etiological or risk factors in patients who suffered from thrombosis. Initial interest in these states of risk dates back to the 1960s with the identification of the relationship of lupus anticoagulant to thrombosis and has proceeded to the understanding of the molecular aspects of several of these conditions. These are addressed later in the discussion of inherited and acquired thrombophilia.

Egeberg described a Norwegian family suffering from thrombotic diatheses linked to a congenital deficiency of antithrombin III (AT) in 1965.¹ Since then, rapidly progressive discoveries of the association between thromboembolism and alterations in factors that inhibit coagulation ensued. Both specific inherited and acquired abnormalities have been identified that are most directly associated with thromboembolic disease in patients younger than 40 years of age. In patients older than 40, malignant neoplasms are commonly the etiological stimulus.^{2,3} In the discussion that follows, risk factors for thrombosis are described individually, but the development of thrombosis is a complex process involving the vascular endothelium, platelets, the flow of blood, and the interactions of coagulation factor—all with their endogenous regulators—as a composite system. Although some conditions exist that are

sufficient to cause disease alone, most inherited and acquired risk factors compound one another to create an optimal environment for thrombosis.

Hypercoagulable states have a considerable effect on society, both in the form of primary pathological thromboses (idiopathic and unprovoked) and secondary to underlying medical conditions (e.g., cancer) and transient states (e.g., long-haul travel) leading to thrombotic complications (provoked). The annual incidence of **venous thromboembolism (VTE)** has been increasing over time, and it has a first-time incidence of approximately 130/100,000 people/year in the United States.³ When one couples the incidence of PE with the thrombotic aspect of arterial disease as manifested by stroke, myocardial infarction (MI), and peripheral vascular disease (PVD), thrombosis becomes overwhelmingly the most common mechanism of death encountered in Western countries.

This chapter explains the regulation of coagulation and fibrinolysis, inherited thrombophilia, acquired thrombotic disorders, and other conditions associated with thrombosis. It presents a diagnostic approach to thrombosis, identifies relevant issues in the laboratory testing of patients with thrombosis, and describes the use and monitoring of anticoagulant therapy. Also included is a discussion on thrombophilia caused by abnormalities in the plasma and the laboratory identification of these abnormalities.

Regulation of Coagulation and Fibrinolysis

The regulation of hemostasis is complex and involves the interaction of many components of the coagulation system. In the normal physiological state, procoagulant and anticoagulant systems are in equilibrium. This balance may be tipped in either direction by a change in physiological or clinical circumstances. Because the concentration of regulatory and procoagulant proteins varies, a patient may vacillate among a prothrombotic, an anticoagulated, and a balanced state. Endothelial cells (and subendothelial structures), platelets, procoagulant factors that participate in the generation of thrombin, natural inhibitors of coagulation (plasma components), and the fibrinolytic system all play important roles in maintaining this equilibrium.

Role of Endothelium

The endothelium plays a key role in the regulation of hemostasis and has anticoagulant and procoagulant functions. The dual roles lie in the needed properties of maintaining a fluid liquid physiological state as well as aiding instant clotting in traumatic situations to prevent exsanguination. As arteries become smaller in their path through the tissue, a given volume of blood is forced into sequentially narrower spaces (the surface area to volume ratio increases) to allow the exchange of oxygen from the red blood cell to the tissues. The peak of this activity is reached in the capillary bed where, at a vessel diameter as small as 3 μm , the surface area to volume ratio approaches 1,000 $\text{mM}^2/\mu\text{L}$.⁴

Imagine spreading 1 mL of liquid uniformly over the surface of an average game table, while allowing no cell or molecule to pass through a capillary without contacting the

endothelial cells lining the vessel. As a result, opportunities for ligand–receptor interactions are provided and, if needed, endothelial cell activation resulting in a slowing of velocity within these areas that allows for an increased chance of unnecessary coagulation. In addition, as in rivers, both large- and medium-diameter vessels have slower blood flow near the vessel wall, creating a need for endothelial cells to have anticoagulant properties along the surface to prevent pathological clot formation. However, if the lining of the vessel is breached, agonists contained in the damaged endothelial cell stimulate immediate hemostatic mechanisms that localize clot formation to the area of trauma to prevent excessive blood loss and thus the cell contains procoagulant properties when needed.

Anticoagulant Role

In the normal state, intact endothelium physically prevents adhesion of platelets to underlying collagen, preventing an initiating event of platelet activation. Endothelial cells discourage platelet aggregation by the continual release of **prostacyclin (PGI₂)** and **nitric oxide (NO)** (both potent aggregation inhibitors). A product of arachidonic acid metabolism, the PGI₂ molecule is the most important natural inhibitor of platelet function. The endothelial cell affects secondary hemostasis by binding the endogenous anticoagulants, **thrombomodulin** and heparin-like molecules, to its surface, thereby inhibiting the coagulation cascade (described in more detail later). Lastly, endothelial cells synthesize **tissue plasminogen activator (tPA)**, which by promoting fibrinolytic activity is instrumental in the degradation of any wayward fibrin deposits on endothelial surfaces.

Prothrombotic Role

When disruption of endothelial cells occurs, platelets adhere to the newly exposed subendothelial collagen, become activated, and secrete substances to recruit and activate additional platelets. The cells synthesize von Willebrand factor (vWF), which is the bridging molecule essential for this platelet adhesion to collagen. vWF is packaged in cytoplasmic secretory granules called Weibel-Palade bodies. It also serves as a carrier molecule for FVIII. This coupling of vWF to FVIII serves two functions: (1) protection of factor VIII from degradation and (2) localization of FVIII to the area of trauma. The endothelial cell also expresses binding sites for activated FIXa and Xa on its surface, further localizing coagulation activity to the site of injury while producing **tissue plasminogen activator inhibitor (TPAI)** to block degradation of a newly formed clot. Interestingly, endothelial cells can be induced by various cytokines and endotoxins to produce tissue factor (also known as thromboplastin), which initiates coagulation through the activation of FVII. This is a mechanism that may explain the procoagulant state seen in septic and neoplastic conditions.

Platelets

Platelets demonstrate activation in response to trauma to the vessel wall. Platelet activation has three steps: (1) adhesion, (2) secretion, and (3) aggregation (see Chapter 25). Adhesion to the underlying collagen, which anchors the activity to the needed area and stimulates a change in platelet shape, defines

the first step of platelet activation. Functional aspects of platelet shape change lie in the rearrangement of the phospholipid platelet membrane. This not only provides a surface for the surface-dependent component of coagulation (contact factors, which regionalizes the coagulation enzymes (contact enzyme–substrate encounters) but also exposes binding sites for platelet-to-platelet binding (aggregation). The catalytic ability of the platelet surface may in part explain why thrombin generation accelerates in the presence of platelets.

The second step of platelet activation is the secretion of agonists that function in recruiting additional platelets and promote further adhesion and aggregation. The platelet release reaction (secretion) contributes to thrombogenesis through the release of the alpha (α) granule contents in response to various stimuli (e.g., thrombin). These granules are visible via light microscopy and contain the platelet-specific proteins, **platelet factor 4 (PF4)**, **beta (β)-thromboglobulin**, **platelet-derived growth factor**, and a variety of other proteins, including fibrinogen, fibronectin, FV, FVIII, and vWF. These contribute to platelet aggregation and thrombin generation, as well as local platelet adhesion. The other type of storage granule, called dense bodies, are visible only via electron microscopy and contain ADP, ATP, calcium, histamine, serotonin, and epinephrine.

The third step in platelet activation is aggregation, which is linked through fibrinogen and creates the end product of primary hemostasis: the platelet plug (the main physical barrier). These three steps of platelet activation are the basis of the platelet function assays. How platelets respond enables assessment of the etiology of platelet disorders.

Procoagulant Factors and Thrombin Generation

Recall that endothelial cells express binding sites for activated FIXa and Xa. They also provide vWF, which bridges platelets to collagen and facilitates efficient clotting by binding and stabilizing FVIII, localizing this powerful promotor of clot formation to the required area of hemostasis. The resultant environment of activated platelets and clotting factors generates thrombin (FIIa) through both the extrinsic and intrinsic pathways of the coagulation cascade. **Thrombin** then is principally responsible for the resultant generation of fibrin monomers, which become the insoluble fibrin strands needed for blood clot formation. Thrombin is the last and most important enzyme of the clotting cascade. The active site of the enzyme is buried deep within a groove that is protected by surrounding structures.

Selective access accounts for the specificity of the enzyme. In addition, thrombin has two positively charged regions that are sites for macromolecule ligand binding (exosite I and exosite II). Exosite I is the binding site for the thrombin receptor, fibrinogen, FV, protein C (PC), and thrombomodulin. Owing to its versatility, thrombin is an extremely interesting enzyme within the coagulation system. The activities of thrombin include:

- It proteolytically cleaves fibrinogen to produce two molecules of fibrinopeptide A and two molecules of fibrinopeptide B from the Aα and Bβ fibrinogen chains, respectively. This results in the conversion of fibrinogen

- to a fibrin monomer. The released fibrin monomers spontaneously polymerize to form fibrin.
- It converts FXIII (in plasma and platelets) to an active transglutaminase, which cross-links fibrin with covalent amide bonds that render the fibrin insoluble in order to become a physically binding structure.
- It activates the procoagulant FV, VIII, and XI, to participate in amplifying its own generation.
- It also binds platelets at low concentrations and initiates shape change, aggregation, and secretion promoting primary hemostasis.
- It participates in self-regulation by binding to thrombomodulin, which then activates PC and PS to inhibit excessive thrombin formation.

The complexity of the thrombin molecule is demonstrated by its participation in self-regulation, and although it is generally considered a powerful procoagulant, it has the capacity to become an anticoagulant.

Natural Inhibitors of Coagulation Factors (Plasma Components)

Suitable activation of coagulation requires a balance between procoagulant and anticoagulant proteins in blood. Loss of this balance may lead to hemorrhage or thrombosis.

Normal plasma contains a sophisticated system of serine protease inhibitors capable of inhibiting many of the activated proteases generated during coagulation that slow the generation of thrombin (Fig. 29-1). A number of these protease inhibitors are members of the serine protease inhibitor (SERPINs) superfamily.⁵ Anticoagulant SERPINs include antithrombin (AT), Heparin Cofactor II (HCII), protein C inhibitor (PCI), α_1 -antitrypsin (A1AT), and protein Z-dependent protease inhibitor (PZPI).⁶ The anticoagulant SERPINs are present in the plasma; however, PN1 is stored in the alpha granules of platelets and is released upon platelet activation.⁵

A number of plasma anticoagulants supplement the inhibitory action of these SERPINs. This includes activated protein C (aPC) and its essential cofactor protein S, which inhibit FVa and FVIIIa. In addition, **Tissue factor pathway inhibitor (TFPI)** is the primary inhibitor of the TF:FVIIa complex. Table 29-1 lists the serine-protease inhibitors and their targets.⁶

Antithrombin (Antithrombin-III)

According to the current international nomenclature, AT-III is renamed antithrombin (AT). AT combines with heparin as a cofactor to become the most powerful anticoagulant in the

TABLE 29-1 Serine-Protease Inhibitors (SERPINs) and Their Targets

Serpin	Targets
α_1 -Antitrypsin (A1AT)	FXa; FXIa
Antithrombin (AT)	Thrombin(FIIa) FXa, FIXa, FXIa; PKa (protease kallikrein)
Heparin Cofactor II (HC-II)	FIIa; FXa
Protein C inhibitor (PCI)	Activated protein C (aPC); FIIa; FXIa; PKa; FXa
Protein Z-dependent protease inhibitor (PZPI)	FXIa; FIXa; FXa
SERPINS	INVOLVED IN FIBRINOLYSIS
α_2 -Antiplasmin	Plasmin
Protease nexin-1 (PN-1)	Plasminogen activators, Plasmin, thrombin
C1- inhibitor (C1-INH)	Plasminogen activators, Plasmin, FXIIa, FXIa, PKa

circulation.⁶ On serum protein electrophoresis, it is an α_2 -glycoprotein composed of a single chain of 432 amino acids with a molecular mass of 58 kDa and an in vivo half-life of approximately 2 to 3 days. AT is synthesized by the liver and belongs to the serine protease inhibitor (serpin) superfamily. Normal plasma levels are approximately 120 mcg/mL.⁶ AT is a major inhibitor of thrombin (FIIa) and factor Xa. Other coagulation factors such as FXIIa, XIa, and IXa are also inhibited but to a lesser extent.⁶

Normally, AT is a relatively weak inhibitor of the serine proteases, but it is activated in vivo by heparin released from granules within mast cells and heparan sulfate, a glycosaminoglycan (GAG) that lines the endothelial layer.⁵ Heparin, which is highly negatively charged, interacts with a short pentasaccharide domain in the AT molecule containing a high density of basic amino acids, particularly lysine. Because of the conformational change in the AT molecule precipitated by the binding of heparin, an arginine residue is made readily available to the active site of a serine protease. A locked complex that blocks enzymatic activity is formed between AT and the factor, tying up the availability of the protease. As the complex forms, the heparin molecule disassociates and is ready to react with another AT molecule. Therefore, heparin administered even in small doses converts AT from a slow, relatively ineffective inhibitor to a fast, effective one. Longer chain heparins, such as those found in unfractionated heparin (UH), inhibit through both approximation (binding and linking the target serine protease to AT via a second heparin binding site) inhibiting thrombin, as well as the short-chain pentasaccharide-induced conformational change, inhibiting FXa.⁵ The conformational change due to this pentasaccharide sequence alone accelerates by several hundred-fold the inhibition of FXa, employed by low molecular weight heparin (LMWH) and fondaparinux (a synthetic pentasaccharide).⁷

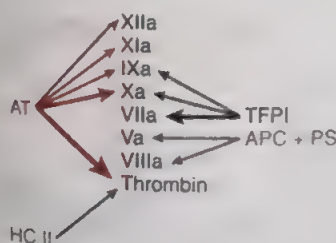


FIGURE 29-1 Physiological inhibitors of coagulation. AT = antithrombin; APC = activated protein C; XIIa = activated factor XII; HC-II = heparin cofactor II; PS = protein S; TFPI = tissue factor pathway inhibitor.

Thrombin/Thrombomodulin Interaction

Thrombomodulin (TM) is a transmembrane glycoprotein synthesized mainly by endothelial cells with a crucial role in the regulation of coagulation. When thrombin is generated at a remote location and released into the circulation, it comes into contact with and quantitatively binds to the TM on the surface of the endothelium. When bound, it changes its enzymatic specificity, no longer recognizing fibrinogen as a substrate and, paradoxically, acts on PC, generating APC. APC, in the presence of its cofactor, PS, forms the APC proteinase complex. This complex has powerful anticoagulant effects by cleaving FVa and FVIIIa and promoting fibrinolysis by inactivating plasminogen activator inhibitor-1 (PAI-1).⁶ The APC–proteinase complex constitutes, on a mole for mole basis, the most active inhibitor of plasma coagulation identified after coagulation⁵ (Fig. 29–2). Therefore, as previously mentioned, thrombin indirectly possesses antithrombotic activities, limiting the extent of its own generation. Soluble TM in plasma also functions as an anti-inflammatory molecule, making recombinant TM a useful preparation for patients with inflammatory disorders such as disseminated intravascular coagulation (DIC).⁸

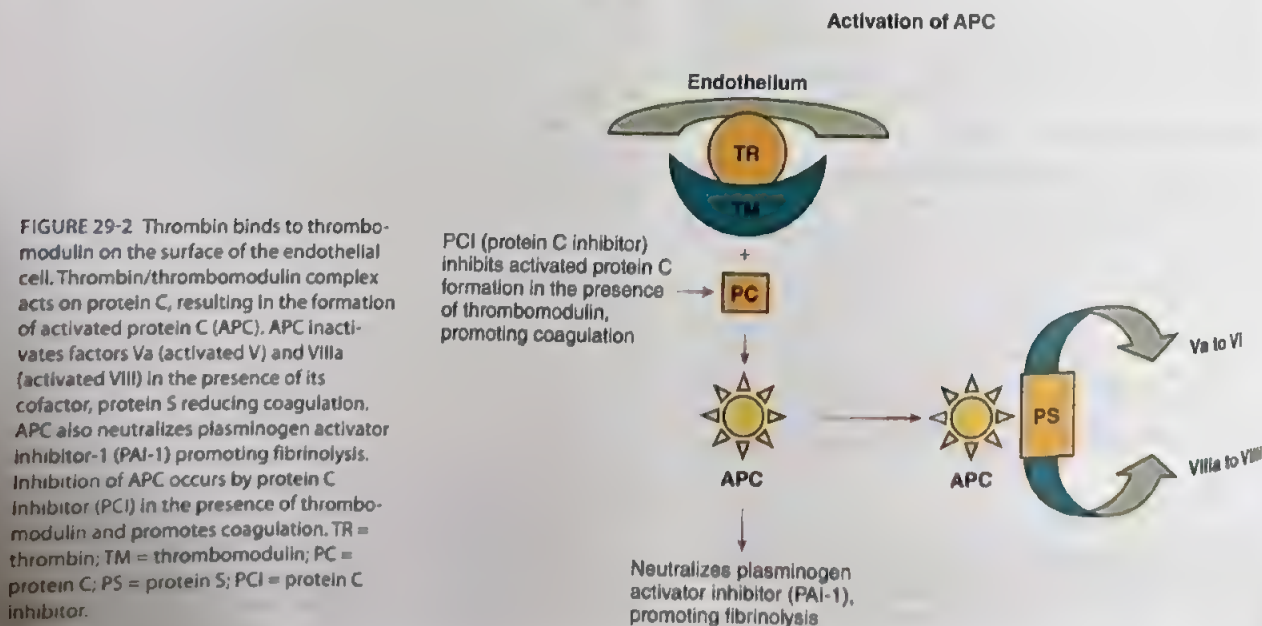
Protein C and S System

PC is a vitamin K–dependent zymogen with anticoagulant properties that, once activated by the TM-altered thrombin molecule, proteolytically degrades FVIIIa and FVa. PC is a glycoprotein with heavy and light chains linked by a disulfide bond with a molecular weight of 62 kD. The anticoagulant role of PC is also described under thrombin/TM interaction, previously, and is depicted in Figure 29–2.^{9,10}

Thrombin/TM activation of PC is modulated by activated FV (FVa) concentrations. FVa concentrations greater than a certain level enhance the activation rate of PC, whereas lower concentrations inhibit PC activation.¹⁰ This provides

a necessary feedback mechanism allowing FVa activation to occur (favors coagulation) until the level becomes high enough to necessitate control of the process (to avoid hypercoagulation) through PC activation.¹⁰ PC function is significantly enhanced in the presence of its cofactor, PS. PS, PC function is inadequate to control the quantities of thrombin.

In the presence of calcium, PS binds to the phospholipid surface of endothelial cells and activated platelets and associates with APC. This localizes the PS–APC complex at the needed site of clot formation. PS is a vitamin K–dependent protein that is produced in the liver, as well as in megakaryocytes and endothelial cells. PS is a single chain with a molecular mass of 69 to 84 kD.⁵ It is unique among the vitamin K–dependent coagulation proteins in that it is not the zymogen of a serine protease. Plasma PS circulates in two forms: one is bound with a 1:1 ratio to the C4b-binding protein (C4bBP) from the complement system, and the other is free. The free PS form represents about 40% of total PS in normal individuals and is the only functional form of PS. Platelets also contain PS; therefore, the activation of platelets provides not only a surface for procoagulant reactions but also a cofactor for eventual control of thrombin generation. Decreased PS synthesis is seen in many conditions, the most common being oral anticoagulant therapy; however, vitamin K deficiency, liver disease, chemotherapy, and L-asparaginase therapy are also known etiologies.¹¹ Consumption of all factors including PS can occur with acute thrombotic events or disseminated intravascular coagulation (DIC). The carrier molecule C4bBP acts as an acute phase reactant with fluctuating concentrations associated with inflammatory states. An increase in C4bBP correspondingly increases the percentage of bound PS antigen and a relative decrease in free PS antigen and therefore PS activity. Conditions that are associated



with an increase in C4bBP concentrations are pregnancy, oral contraceptive use, diabetes mellitus, and systemic lupus erythematosus. Free PS antigen and activity are also often decreased in nephrotic syndrome.¹¹

Protein C inhibitor (PCI) inhibits the thrombin-TM complex, which indirectly inhibits APC formation and is enhanced in the presence of heparin.^{5,12}

Tissue Factor Pathway Inhibitor

The **tissue factor pathway inhibitor (TFPI)** circulates and is associated with a lipoprotein that inhibits plasma coagulation in a dual manner: (1) it serves to bind the activated form of either FX or IX, thereby inhibiting their enzymatic activity and (2) the TFPI-FXa or IXa complex then binds to the membrane-bound FVIIa-tissue factor complex, competitively inhibiting further activation of FX or FIX through the extrinsic pathway. PS also acts as a cofactor for TFPI function.¹³ These actions are depicted in Figure 29-3. The role of TFPI in clinical thrombosis has been studied in animal models. The absence of circulating TFPI activity has been shown to result in the reversal of aspirin inhibited intravascular thrombus formation. TFPI plasma levels appear to be acutely consumed in acute myocardial infarction from intracoronary thrombosis. Most intriguing is recent data regarding arterial wall gene transfection with that coding TFPI with promising therapeutic implications in the prevention of intravascular thrombus formation. Certainly, TFPI is an important component of dysregulated coagulation and may represent a pivotal protein in novel therapeutic interventions.^{14,15}

Fibrinolytic System

The fibrinolytic system in vascular hemostasis is critical for controlling the amount of clot formation and resolving clots no longer needed. The plasminogen inhibitors contribute to clot stability by blocking premature dismantling of the thrombus. Among the components of the fibrinolytic system are plasminogen-plasmin and the activator system: urokinase-plasminogen

activator (uPA) and tissue-type plasminogen activator (tPA), which convert plasminogen into the functional form, plasmin. The major inhibitors are α_2 -antiplasmin and plasminogen activator inhibitors 1 and 2 (PAI-1, PAI-2).⁶ Physiological fibrinolysis results in the proteolytic degradation of polymerized fibrin. The central reaction in this system is the conversion of a proenzyme, plasminogen, to the proteolytic enzyme plasmin. Plasminogen is a 90 kD glycoprotein synthesized in the liver with a circulating half-life of 2 to 3 days.⁵ Plasminogen circulates both in the free state or bound to histidine-rich glycoprotein (HRGP), α_1 -antiplasmin, or fibrinogen itself. The physiological activation of plasminogen is achieved through the extrinsic pathway initiated by tPA release from the endothelial cells after stimulation and by the intrinsic pathway through a FXIIa-dependent activator and urokinase.

A specific group of proteins control fibrinolysis. They are serine protease inhibitors and are members of the serpin superfamily. They are structurally homologous to many inhibitors of the serine proteases of the coagulation system (Table 29-1). Plasmin activity is regulated and inhibited by a number of plasma proteins such as α_2 -antiplasmin, α_2 -macroglobulin, α_1 -antitrypsin, AT (especially in the presence of heparin), and C1 esterase inhibitor.⁵ Physiologically, α_2 -antiplasmin, the main plasmin inhibitor, is a 60 kD glycoprotein and circulates in the plasma at a concentration of 1 μ M.⁵ Its properties include:

- Direct inhibition of plasmin
- Interference with absorption of plasminogen to fibrin
- Susceptibility to FXIII-catalyzed cross-linking of antiplasmin to fibrin that incorporates the enzyme into the structure of the clot providing stability by inhibiting intrathrombus plasmin activity

The combined effects of these three characteristics render α_2 -antiplasmin much more specific and effective in inhibition of fibrinolysis than any of the other major inhibitors.

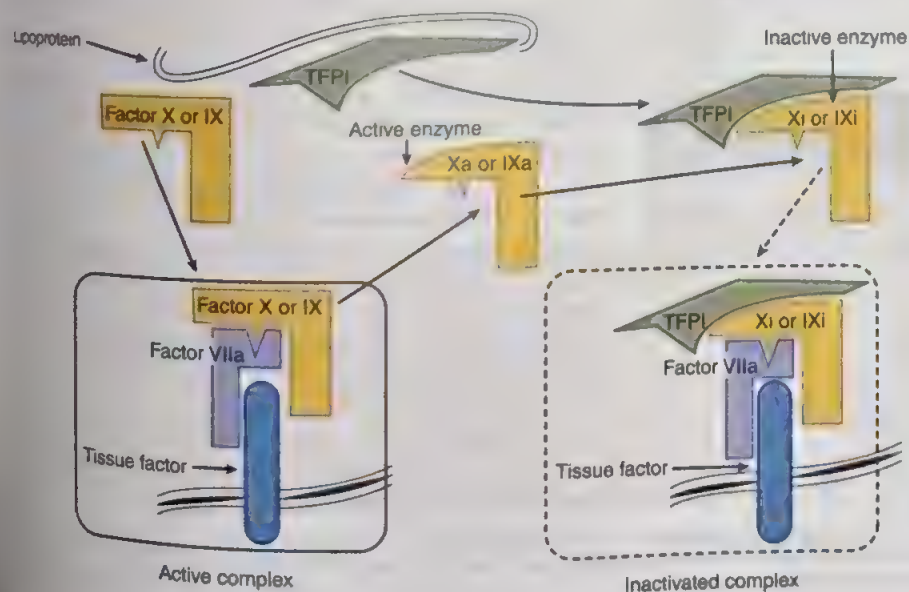


FIGURE 29-3 Shown here is the interaction of tissue factor pathway inhibitor (TFPI) with factors VII, IX, and X. (Courtesy of John D. Olson, MD, PhD; Department of Pathology, University of Texas Health Science Center, San Antonio, TX.)

Plasminogen activators such as uPA and tPA are inhibited by a number of specific proteins. PAI-1 and PAI-2 are found in plasma and urine² (Table 29-2). These inhibitors are nonspecific in nature and deactivate enzymes extrinsic to the coagulation system. The most important inhibitor in the plasminogen activator system is PAI-1, the primary fast-acting serpin inhibitor of tPA. The physiological plasma concentration of PAI-1 varies widely from 0 to 60 ng/mL, with an average range of 5 to 20 ng/mL, and many patients with myocardial infarction have elevated levels.^{3,6,11} Because the role of this enzyme is to dampen the degradation of a clot, there has been interest in defining a role in myocardial infarction and stroke, especially in atypical patients (younger than 45 years of age). Although a case control study reported a correlation of younger patients with myocardial infarction and a PAI-1 genetic polymorphism, subsequent prospective studies did not support this finding.

ADVANCED CONTENT

Another enzyme under investigation is thrombin activatable fibrinolysis inhibitor (TAFI), also known as procarboxypeptidase B. This clot-stabilizing 55 kD glycoprotein is synthesized in the liver and platelet alpha granules and circulates in plasma bound to plasminogen. TAFI cleaves C-terminal lysine groups, essentially removing the binding sites for plasminogen and tPA to fibrin strands. Its role in hypercoagulable states and inflammation continues to be investigated.¹⁶

The fibrinolytic system is as complex as the coagulation system. The cause-and-effect relationship between the frequency of deficiencies in the physiological fibrinolytic system and the occurrence of thromboembolism is not fully understood. However, it seems the most common abnormality is the presence of excess PAI-1 leading indirectly to a decreased functional availability of tPA, which results in a hypercoagulable state.

TABLE 29-2 Tissue Plasminogen Activator (tPA) Inhibitors

Characteristics	Types	
	PAI-1	PAI-2
Origin	<ul style="list-style-type: none"> • Plasma • Platelets • Endothelial cell • Granulocytes 	<ul style="list-style-type: none"> • Placenta • Leukocytes • Macrophages
MW (daltons)	54,000	47,000
Inhibitory effect	TPA	TPA
Concentration	UK	UK
	0–1.3nM	2 μ M (3rd trimester)

UK = urokinase; MW = molecular weight; nM = nanomoles; μ M = micromoles.

CRITICAL THINKING QUESTION

29-1 Why would liver disease affect coagulation and fibrinolysis? See answers to all Critical Thinking Questions at the back of this book.

Inherited Thrombophilia

Inherited thrombophilia is a group of congenital hematologic disorders that includes a variety of hypercoagulable states which usually present clinically as venous and/or arterial thrombosis. Hypercoagulability is usually defined as an alteration of the blood coagulation mechanism that predisposes one to thrombosis. For thrombosis to manifest clinically, more than one and presumably several risk factors need to be present simultaneously to overcome the natural protective processes in the blood.

Activated Protein C Resistance

The exogenous addition of APC to a test tube of normal plasma creates an anticoagulant effect that can be measured as a prolongation of the activated partial thromboplastin time (aPTT). The absence of this anticoagulant response reflects the decreased ability of APC to inactivate FVa and results in a thrombophilic state called **APC resistance (APC-R)**.¹⁷ The concept of APC-R was first described by Dahlback and associates in 1993.¹⁸ In the majority of cases, APC-R is the result of a genetic defect. The predominant etiology of APC-R is a defect in the *F5* gene involving the point mutation at codon 506 of exon 10, which substitutes a glutamine residue for the wild-type arginine amino acid (R506Q), factor V Leiden (FVL).¹⁹ This codon is the site where APC cleaves and inactivates FVa. The activity of FV and its role in coagulation are not affected. The mutated FVa is resistant to cleavage by APC and results in extended procoagulant activity; therefore, it increases the risk of pathological thrombus formation.¹¹ Additional rare mutations leading to APC-R as well as acquired causes (e.g., autoantibodies) have also been identified.

Among Caucasians, APC-R is the most common risk factor associated with inherited venous thrombosis, and the most common cause is FVL mutation, with autosomal-dominant inheritance.²⁰ The heterozygous state is relatively common in Western countries and occurs in approximately 2% to 7% of the general population. The prevalence in Hispanic, African American, Asian, and Native American populations appears to be low. For a first episode of VTE, the thrombotic risk for patients carrying heterozygous FVL mutation is increased approximately 4- to 6-fold, while the risk for homozygous patients is around 7- to 20-fold.²¹ For recurrent VTE, the increased relative risk for both heterozygous and homozygous mutations may be slightly increased compared with persons without a defined thrombophilia but is not clearly significant (1.1–1.8).^{21,22}

FVL-associated thrombotic risk is dependent on the underlying clinical setting. Although oral contraceptives and pregnancy may increase the risk for venous thrombosis in women with the mutation, the increased thrombotic risk in patients with preexistent cancer or recent surgery cannot be demonstrated. There is no convincing data that FVL

mutation confers an increased risk for arterial thrombosis. Data for FVL as an independent risk factor for myocardial infarction are controversial.^{21,22,23}

Most of the screening tests for APC-R are functional aPTT-based assays.²⁴ They can be used as a screening method to identify patients in need of the confirmatory FVL molecular test. The functional APC-R assay is performed by measuring an aPTT both in the absence and then the presence of a standardized amount of APC, with a calculation of the resultant ratio (the APC ratio, Fig. 29-4). In a normal person, the addition of APC to plasma induces a prolonged aPTT because APC cleaves and inactivates FVa and VIIIa. In patients with APC-R, the aPTT is relatively shorter than in normal plasma. FVa is not cleaved and therefore is not inactivated. The test results can be interpreted by comparing the ratio to the normal range or by normalizing it to the APC-R ratio obtained using normal pooled plasma.²⁴ By diluting the patient's plasma with an excess of FV-deficient plasma, the sensitivity and specificity of the aPTT-based APC-R assay can be increased and allow the analysis of plasma from patients who are taking warfarin or have other factor deficiencies without interference.²⁴ FV-deficient plasma is made of normal plasma with all the moieties needed for coagulation except FV (which the patient's plasma must contribute to the mixture) and thus normalizes the concentrations of other plasma proteins involved in the formation and regulation of thrombin. The limitation

of this aPTT-based functional assay (and, in fact, all aPTT-based assays) is that the results are unreliable when the test is performed on patients with an inhibitor. For example, patients treated with heparins and direct oral anticoagulants (DOACs) or a lupus anticoagulant can artificially prolong the baseline aPTT even with repletion of coagulation proteins. The aPTT test, however, may be performed during an acute thrombotic episode.

The genetic test for FVL mutation is often referred to as the FVL assay.²⁵ In this test, the genomic DNA is isolated from blood mononuclear cells. Using allelic discrimination methodology by polymerase chain reaction (PCR), the sample DNA is analyzed, allowing specific genotypes to be identified (normal, heterozygous, or homozygous).²⁵

The genetic test is usually performed to confirm the FVL mutation and has several advantages over plasma-based functional assays. The genetic test is not affected if the patient is receiving anticoagulants or has plasma inhibitors (such as lupus anticoagulant). It does not have a threshold range as does the functional plasma-based assays, and it can reliably differentiate between heterozygous and homozygous states.²⁵

One of the approaches in diagnosing patients with FVL is to perform an aPTT-based functional assay for APC-R. Patients with low APC-R ratios (results may vary among different laboratories) should be genotyped for the FVL mutation.²⁴ Even in laboratories in which there is an excellent

$$\text{APC-R RATIO: } \frac{\text{Patient clot time APC}}{\text{Patient clot time C}} \quad \text{Normal ratio is } >2.0$$

$$\text{Example case \#1} \quad \frac{84 \text{ sec}}{35 \text{ sec}} = \text{ratio of patient is } 2.4$$

$$\text{Normal pool plasma} \quad \frac{70 \text{ sec}}{35 \text{ sec}} = \text{ratio of normal pooled plasma is } 2.0$$

$$\frac{\text{Patient ratio}}{\text{Normal pooled plasma ratio}} = \frac{2.4}{2.0} = \text{normalized ratio is } 1.2$$

(Final ratio of less than 0.9 is abnormal)

Interpretation: The APC-R ratio in Case 1 is 1.2; the test is negative for APC resistance.

$$\text{Example case \#2} \quad \frac{59 \text{ sec}}{35 \text{ sec}} = \text{ratio of patient is } 1.7$$

$$\text{Normal pool plasma} \quad \frac{70 \text{ sec}}{35 \text{ sec}} = \text{ratio of normal pooled plasma is } 2.0$$

$$\frac{\text{Patient ratio}}{\text{Normal pooled plasma ratio}} = \frac{1.7}{2.0} = \text{normalized ratio is } 0.85$$

(Final ratio of less than 0.9 is abnormal)

Interpretation: The APC-R ratio in Case 2 is 0.85; the test is positive for APC resistance and therefore PCR tests for factor V Leiden mutation should be conducted for this patient.

Note The sensitivity of the test is increased by comparing the ratio to the normal range or by normalizing it to the APC resistance obtained using normal pooled plasma

FIGURE 29-4 Data on the functional assay for activated protein C are shown here. This is an aPTT-based assay, and the APC-R ratio can be calculated by measuring the patient clot time with and without activated protein C (APC) in the presence of CaCl₂. In a normal person, the ratio is more than 0.9. In case 1, the final ratio is 1.2 and thus the test is negative for activated protein C resistance (APC-R). In case 2, the ratio is less than 0.9 and the test for APC-R is positive. In case 2, therefore, the patient should be tested for factor V Leiden mutation by polymerase chain reaction (PCR). APC-V = activated protein C-factor V ratio.

concordance between APC-R assays and the results of FVL mutation assays, some patients with a low APC-R ratio and negative genetic results can still be identified.²⁵ The significance of this finding is uncertain and may be related to mutations other than FVL or an acquired APC-R.

Protein C Deficiency

PC is vitamin K-dependent glycoprotein that is synthesized by the liver. Hereditary deficiency of PC shows an autosomal dominant inheritance pattern. The prevalence of PC deficiency is 0.2% to 0.5% of the general population.²⁶ For a first episode of VTE, the thrombotic risk for patients with PC deficiency is increased approximately 10-fold.²⁶ For recurrent VTE, the increased relative risk is slightly increased (1.8) compared with persons without a defined thrombophilia. Homozygous individuals have a marked tendency for recurrent venous thrombosis and PE, neonatal purpura fulminans, and warfarin-induced skin necrosis.²⁷ Interestingly, heterozygotes commonly do not manifest thrombosis unless concomitant additional risk factors exist.

Warfarin-induced skin necrosis has been associated with patients who have PC deficiency.²⁷ It occurs during the first few days of warfarin therapy. Warfarin ingestion results in a decrease in vitamin K-dependent factors (II, VII, IX, X, PC, and PS). Because PC has a short half-life (6 hours) compared with other vitamin K-dependent factors, the decrease in PC level occurs before the decrease in FII and X levels. This causes a temporary imbalance between procoagulant and anticoagulant factors, resulting in a transient hypercoagulable state that can lead to thrombosis and subsequent skin necrosis.

Infants with inherited homozygous PC deficiency may develop thrombosis of small capillaries including cerebral vessels and laboratory evidence of disseminated intravascular coagulation (DIC). The condition is usually fatal and is called *neonatal purpura fulminans*.²⁸ It is important not to misinterpret the physiologically low level of PC in a normal newborn, which may be near zero in a sick preterm infant.²⁸ The level usually rises rapidly after birth.

The functional PC activity assay is used as the screening assay for PC deficiency. The antigenic assay is used to determine the mechanism of the deficiency (decreased production or abnormal protein) and is rarely necessary in diagnosis. Based on immunological and functional assays, two subtypes of heterozygous PC deficiency have been described.²⁷ Type I (quantitative) deficiency is the most common, which shows a reduction in the activity due to a concomitant decrease in the amount of antigen (to approximately 50% of normal).^{26,27} Type II (qualitative) deficiency (further subsequently subtyped as IIa and IIb, described later) is less frequent, and like type I, shows reduced functional activity but can be differentiated by the measurement of normal antigen levels.^{26,27} This type is a result of defective molecular mechanisms that allow expression of a protein that has lost functional capacity.²⁷

The chromogenic PC assay is the recommended first assay given its improved specificity over the clot-based assays.²⁶ For both, PC is activated in the presence of the specific activator extracted from snake venom (Southern Copperhead viper venom, Protac®). Chromogenic assays detect

enzymatic cleavage of a small synthetic substrate, allowing identification of Type I and Type IIa PC deficiencies.²⁶ Clot-based assays (aPTT or RVV) work by inhibiting the cleavage of FVa and VIIIa leading to prolonged clot times and, as such, also identify defective interactions with phospholipids, calcium ions, FVa and VIIIa, or PS (Type IIb) (Table 29-3). While the clot-based assay carries the advantage of identifying an additional subset of cases, numerous interferences, including high levels of FVIII (greater than 250%), anticoagulant therapy (DOACs, heparin), and lupus anticoagulants limit its utility as a first line assay. Both assays may be limited by sample activation, as well as hemolysis, icterus, and lipemia (HIL).²⁶ The procedures used for antigenic assays are most commonly enzyme-linked immunosorbent assay (ELISA), historically electroimmunodiffusion (Laurel rocket electrophoresis), and radioimmunoassay.²⁸

Acquired PC deficiency must be excluded and can be seen in a number of conditions, which include liver disease, DIC, warfarin therapy, severe infection/septic shock, adult respiratory distress syndrome, postoperative states, acute thrombotic episode, and secondary to chemotherapy (e.g., L-asparaginase, methotrexate, cyclophosphamide, and 5-fluorouracil) (Table 29-4). It is important to note that due to the vitamin K dependency of this factor, measurement cannot be taken while on vitamin K antagonists and should be drawn only after an appropriate waiting period subsequent to the cessation of therapy (approximately 4 weeks).

Protein S Deficiency

PS is a vitamin K-dependent nonenzymatic glycoprotein synthesized mainly in the liver but to some extent in endothelial cells, megakaryocytes, and other cells. It exists in an active free form that functions as a cofactor for APC and an inactive form bound to complement C4b-binding protein (C4bBP). The free form accounts for 40% of total PS and has a half-life of approximately 45 hours.²⁹

PS deficiency is inherited as an autosomal dominant disorder. The prevalence of PS deficiency in the general population is approximately 0.1% to 0.7%.^{29,30} For a first episode of VTE, the thrombotic risk for patients with PS deficiency is increased approximately 10-fold. For recurrent VTE, the increased relative risk (1.0) is not clearly increased compared with persons without defined thrombophilia.^{22,24} In contrast to PC deficiency, even heterozygotes have a strong tendency to develop DVT, PE, cerebral and mesenteric thrombosis, superficial thrombophlebitis, and arterial thrombosis and may also develop warfarin-induced skin necrosis. Patients

TABLE 29-3 Classification of Hereditary Protein C (PC) Deficiencies

Subtypes	Antigen	Clot-Based Assay Activity	Chromogenic Activity
I	Low	Low	Low
IIa	Normal	Low	Low
IIb	Normal	Low	Normal

TABLE 29-4 Causes of Acquired Deficiency of Coagulation Inhibitors

Inhibitors	Causes of Acquired Defects
AT	<ul style="list-style-type: none"> • DIC • Liver disease • Nephrotic therapy • Oral contraceptives • L-Asparaginase
Protein C	<ul style="list-style-type: none"> • Oral anticoagulant treatment • DIC • Vitamin K deficiency • After plasma exchange • Postoperative state • L-Asparaginase • Liver disease
Protein S	<ul style="list-style-type: none"> • Oral anticoagulant treatment • Pregnancy • Oral contraceptives • Vitamin K deficiency • Liver disease • Diabetes type I • Acute inflammation • Newborn infants

with homozygous PS deficiency are severely affected and may develop neonatal purpura fulminans just after birth, similar to that seen with PC deficiency.

Both immunological and functional methods are available to measure PS. Reliable measurements of total antigen are performed using radioimmunoassay, latex immunoagglutination assays (LIA), ELISA, or electroimmunodiffusion (Laurell rocket electrophoresis). Functional assays are performed for the quantitative measurement of the free functional PS level based on the ability of PS to serve as a cofactor for the anticoagulant effect of APC inhibition of FVa. This inhibition is reflected by the prolongation of the clotting time of a system that is enriched with FVa, a physiological substrate for APC.

Based on the immunological and functional assays described earlier, three subtypes of PS deficiency have been noted (Table 29-5). The most common, type I (quantitative) deficiency, is associated with decreased activity due to a low antigenic level of total and free PS levels. In type II (qualitative) deficiency, total and free PS levels are normal; however, the functional PS activity is low. These cases are extremely rare, accounting for between 1% to 5% of all PS deficiencies.²⁹

TABLE 29-5 Classification of Hereditary Protein S (PS) Deficiencies

Subtypes	Total PS	Free PS	PS Activity
I	Low	Low	Low
II	Normal	Normal	Low
III	Normal	Low	Low

In type III (quantitative) deficiency, total antigen levels are normal, but free PS antigen and activity levels are low.²⁹ Currently, free protein S antigen assay is the recommended initial assay, despite being normal in type II phenotypes, given its relative insensitivity to preanalytic variables compared with protein S activity assays.^{29,31}

Acquired PS deficiency can be seen in liver disease, DIC, pregnancy, oral contraceptive use, vitamin K deficiency, during an acute thrombotic event, and in therapy with warfarin and L-asparaginase³⁰ (see Table 29-4). C4bBP is an acute-phase reactant, and its increase will proportionately bind free PS; therefore, the amount of free PS may decrease in inflammatory conditions such as systemic lupus erythematosus (SLE), inflammatory bowel disease, pregnancy, and acquired immunodeficiency syndrome (AIDS).³¹ Because PS acts as a cofactor for APC, functional assays may show spurious low PS levels in patients with APC-R.²⁹

Antithrombin Deficiency

Because antithrombin (AT) serves to regulate the most powerful procoagulant, thrombin, a deficiency causes a prothrombotic state.³² Homozygous states are largely incompatible with life except for a rare functional (type II) variant, and the majority of affected individuals are heterozygous with AT levels 40% to 70% of normal.³² It is an autosomal dominant disorder with a prevalence in the general population of approximately 0.02%. For a first episode of VTE, the thrombotic risk for patients with AT deficiency is increased approximately 10 to 30 times.^{31,32} For recurrent VTE, the increased relative risk is slightly increased (2.6) compared with individuals without a defined thrombophilia.³²

The initial clinical manifestation of AT deficiency is commonly between the age of 10 and 50 years (peak 15 to 35), may occur spontaneously in half of the patients, and is characterized by thrombosis of the deep veins of the lower extremities, mesenteric veins, and PE.³³ In the remaining patients, thrombosis may occur in the presence of additional risk factors such as pregnancy, the use of oral contraceptive pills, surgery, or trauma.^{34,35}

Acquired AT deficiency is common, arising from conditions such as decreased synthesis (liver disease or L-asparaginase treatment), consumption (thrombosis, disseminated intravascular coagulation [DIC], surgery) or loss (proteinuria in renal disease), and continuous heparin use³⁶ (see Table 29-4). Mild decreases can be seen with pregnancy, oral contraceptive use, and colitis.³⁶ Before a diagnosis of AT deficiency is rendered in any patient with these conditions, the test should be repeated once the condition is no longer present.

Hereditary AT deficiency may require documentation of a deficiency in a blood relative or genetic testing.

Various methods have been described for the measurement of AT. Immunological assays such as ELISA, rocket immunoelctrophoresis, immunoturbidimetric assays, and radial immunodiffusion are available that measure the antigen levels but cannot be used to detect dysfunctional molecules.³⁶ To detect the qualitative abnormality of the AT molecules, an activity assay is preferred. A known amount

of thrombin or FXa is added to the patient's plasma containing AT in the presence of heparin. The aliquot is removed and added to a specific synthetic chromogenic or fluorescent thrombin substrate and the released compound is measured spectrophotometrically or via fluorometer, respectively.³⁶ A progressive activity assay is an activity assay with two significant alterations: (1) no heparin present in the reagent, and (2) the incubation time is extended.³⁶ Because the reaction time is slower, it allows for measurement of AT activity without contribution from the heparin-binding site (HBS). The progressive activity assay has lower specificity because it may be affected by other inhibitors (e.g., trypsin inhibitor and α 2-macroglobulin).

Based on these assays, two types of AT deficiency can be distinguished phenotypically. In the common type I deficiency, there is a proportionate decrease in both the functional and antigen levels because the deficiency is due to a decreased amount of a functionally sound molecule (quantitative defect). More than 250 mutations in the AT gene *SERPINC1* have been reported.³² Type II deficiency shows a more pronounced decrease in the functional level with a relatively normal antigen level (qualitative or dysfunctional defect) because the molecule is created but does not function properly. Type II is associated with point mutations and can be further subdivided into three subtypes: those characterized by abnormalities primarily affecting (1) the serine protease inhibition site for thrombin/Xa (i.e., the reactive site [RS]), (2) HBS, and (3) those with effects on both protease inhibition and heparin binding (i.e., pleiotropic).^{36,37} The classification of hereditary AT deficiencies is summarized in Table 29-6.

Based on functional assays, the prevalence of thrombosis appears to be different in heterozygous patients with type II defects. Individuals with HBS defects have infrequent thrombotic episodes while, in contrast, those with RS defects sustain venous thrombosis as often as type I patients.³⁷

CRITICAL THINKING QUESTION

29-2 Why are many thrombophilic conditions detected first in young adulthood, especially in women?

TABLE 29-6 Classification of Hereditary Antithrombin (AT) Deficiencies Activity

Subtypes	AT Antigen	AT-Heparin Cofactor	Progressive AT
I	Low	Low	Low
II (RS)	Normal	Low*	Low**
II (HBS)	Normal	Low*	Normal
II (pleiotropic)	Normal/Low	Low	Low

RS: Reactive site; HBS: heparin-binding site

*Differences in thrombin and FXa-based assays may lead to misdiagnosis in some Type II AT deficiencies.

**Except for AT Cambridge II which will be normal.

ADVANCED CONTENT

Prothrombin (F2) G20210A Mutation

Prothrombin (FII) is a vitamin K-dependent factor that is converted to thrombin (FIIa) by FXa in the presence of FVa, Ca^{2+} , and phospholipids. The combination of FVa, Xa, Ca^{2+} , and phospholipids is called the *prothrombinase complex*, a macromolecular enzyme complex in which prothrombin is a substrate and thrombin the product. Hereditary disorders of prothrombin synthesis may result in quantitative defects (decreased production) and qualitative defects (dysfunctional prothrombin molecule). Clinically, both of these defects can result in a bleeding diathesis.

A 21-kb gene present on chromosome 11 codes for the prothrombin molecule.³⁸ This gene consists of 14 exons and 13 introns. A mutation in the prothrombin gene has been described, revealing the presence of a single guanine (G) to adenine (A) mutation at the nucleotide position 20210 to be associated with an elevated prothrombin level and an increased risk of DVT.³⁸

The heterozygous state is relatively common and occurs in approximately 1% to 2% of the general population; homozygous state is rare.³⁸ For a first episode of VTE, the thrombotic risk for patients carrying heterozygous FVL mutation is increased approximately 2 to 4 times, whereas the risk for homozygous patients is approximately 2 to 21 times.³⁹ For recurrent VTE, the relative risk for heterozygous mutations can be slightly increased compared with persons without a defined thrombophilia, but it is not clearly significant (0.7–2.3). The relative risk for homozygous states is uncertain.³⁹

Carriers of this mutation have higher plasma prothrombin levels than those with a normal 20210 genotype. A high level of prothrombin alone is not a reliable marker of disease predisposition, whereas detection of the mutation by genetic testing appears to be a reliable prognostic factor.³⁹

At present, the PCR test for F2 G20210A mutation is available in clinical laboratories. In this test, DNA is isolated from blood mononuclear cells. Using allelic discrimination methodology by PCR, the sample DNA is analyzed, allowing specific genotypes to be identified (normal, heterozygous, or homozygous). Among the Dutch population, the prothrombin gene mutation is the second most common genetic defect (the most frequent being the FVL mutation). Because of the high prevalence of APC resistance (e.g., FVL) and of the prothrombin gene mutation (F2 G20210A), combinations of genetic defects are relatively common. The factor V Leiden and the prothrombin gene mutation G20210A account for about 50% to 70% of the diagnosed genetic thrombophilia.³⁹ Patients with both mutations (double heterozygotes) may have a higher risk of thrombosis.³⁹ However, more studies are needed to improve our understanding of the ethnicity-specific variability of the FVL and prothrombin gene mutations.

Hyperhomocysteinemia

Hyperhomocysteinemia (an elevated plasma level of homocysteine $>15 \mu\text{mol/L}$) is a risk factor for the development of early atherosclerotic vascular disease and venous thrombosis.⁴⁶ Some investigators include hyperhomocysteinemia under acquired disorders, whereas more recent studies suggest that these should be included among the inherited causes of thrombophilia.

ADVANCED CONTENT

Homocysteine is an amino acid derived from metabolic conversion of methionine. It is usually metabolized within the cells to methionine (via the remethylation pathway) and to cysteine (via the transsulfuration pathway).⁴⁶ Remethylation to methionine involves two pathways. In the first remethylation pathway, homocysteine accepts a methyl group from betaine. In the second remethylation pathway, homocysteine accepts a methyl group from 5-methyltetrahydrofolate, with vitamin B_{12} acting as a cofactor.⁴⁶ This pathway is catalyzed by methionine synthase. The transsulfuration pathway is catalyzed by cystathionine β -synthetase with vitamin B_6 as a cofactor (Fig 29-5).⁴⁶

Hyperhomocysteinemia can be inherited or acquired. Inherited forms have genetic defects that involve (1) the remethylation pathway, as a result of a deficiency of methylene tetrahydrofolate reductase (MTHFR), and (2) the transsulfuration pathway, owing to the deficiency of cystathionine β -synthetase.⁴⁶ Multiple MTHFR gene mutations have been described and in general, distribution between populations varies. The mutation of MTHFR gene (alanine substitution to valine at amino acid 677) is more common.^{41,42}

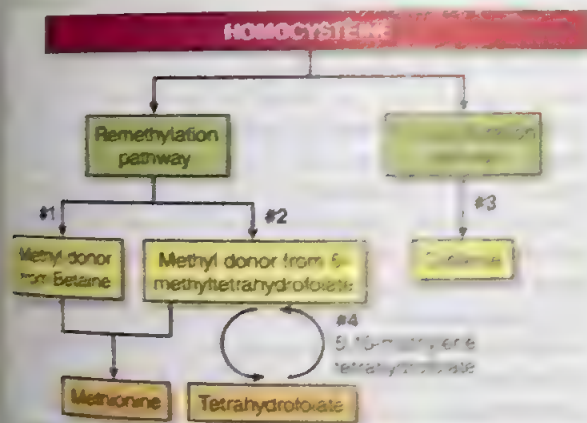


FIGURE 29-5 Homocysteine can be metabolized to methionine via remethylation, and to cysteine via transsulfuration. The numbers represent the enzymes involved in the reactions: 1, betaine homocysteine methyltransferase; 2, methionine synthase in the presence of cofactor vitamin B_{12} ; 3, cystathionine β -synthetase in the presence of cofactor vitamin B_6 ; 4, methylene tetrahydrofolate reductase (MTHFR).

The frequent causes of acquired hyperhomocysteinemia are secondary to deficiencies of folate, vitamin B_{12} , and/or vitamin B_6 , which are cofactors in homocysteine metabolism.⁴²

Possible mechanisms by which hyperhomocysteinemia acts as a thrombogenic and atherogenic risk factor include vascular smooth muscle proliferation, inhibition of endothelial cell growth and intimal thickening, activation of FV, and inhibition of PC activation.⁴⁶ Severe hyperhomocysteinemia is usually secondary to genetic defects and characterized by mental retardation, premature atherosclerosis, venous thromboembolism, and skeletal abnormalities.⁴⁶ Mild to moderate hyperhomocysteinemia can be secondary to genetic or acquired conditions and is an independent risk factor for stroke, MI, and peripheral vascular disease.⁴⁶ Levels of homocysteine can be measured by high-pressure liquid chromatography. A PCR-based genetic test is available to detect the common mutations in the MTHFR gene. However, testing for this genetic mutation is not recommended routinely because it does not represent a significant prothrombotic risk, in the absence of elevations of fasting plasma homocysteine level.⁴⁶ Regardless, ongoing debates exist, arguing the need for testing in obstetric patients with repeated pregnancy losses.⁴³

Tissue Factor Pathway Inhibitor Deficiency

Tissue factor pathway inhibitor (TFPI) may play a significant role in preventing thrombus formation; however, deficiency of this inhibitor may be associated with hormone-induced venous thromboembolism.⁴⁴ There are two TFPI; namely, TFPI 1 and TFPI 2, which are encoded by separate genes.⁴³ Evidence suggests that TFPI 1 and 2 are homologs with different protease inhibitory actions in the coagulation and fibrinolysis pathways.⁴³ The TFPI 1 inhibits factor Xa and IXa.^{43,45} TFPI can be found in endothelial cells, plasma, and platelets. The endothelial cell pool represents the majority of the TFPI. Most of the plasma pool is complexed with various lipoproteins, whereas only 10% of the TFPI is present as a free form and is biologically active.⁴³ The concentration of TFPI is increased in plasma in patients receiving heparin infusion. Recombinant TFPI is now available and has been evaluated in a few studies, with results showing a possible role of TFPI in the interruption of thrombus formation.

Additional evidence suggests that autoantibodies to tissue factor pathway inhibitor (TFPI) and/or antiphospholipid antibodies (aPL) may contribute to upregulation of the tissue factor (TF) pathway of blood coagulation and the development of thrombotic complications in the antiphospholipid syndrome (aPS).^{43,46}

Factor XII Deficiency

FXII is one of the contact factors that initiates the intrinsic pathway of coagulation in vitro. It is also known as Hageman factor, after the patient in whom the deficiency was first identified in Cleveland in 1955.⁴⁶ Patients with FXII deficiency have a prolongation of APTT but no bleeding diathesis.⁴⁷ Instead, several cases with venous thromboembolism and myocardial infarction have been described in FXII-deficient patients.⁴⁷ Mr. Hageman himself died of a PE. Because FXIIa is involved in activating plasminogen, the thrombophilic tendency in FXII deficiency patients has been attributed to reduced plasma fibrinolytic activity.^{47,48} While there does not appear to be an increased incidence of thrombosis in patients

with heterozygous FXII deficiency, severe or homozygous states may lead to increased risk.^{47,49,50}

Dysfibrinogenemia

Dysfibrinogenemia is a condition in which there is a structural or functional abnormality of the fibrinogen molecule, or both. If congenital, the inheritance is autosomal dominant.⁵¹ The clinical presentation is variable and can include bleeding or clotting tendencies. Approximately 60% of the patients are asymptomatic; a bleeding diathesis can be seen in 20% of cases; and 20% of cases may present with recurrent arterial or venous thromboembolism.⁵¹

A number of functional and biochemical defects of the fibrinogen molecule have been described. These include an abnormality in binding of thrombin to fibrinogen, the release of abnormal fibrinopeptides, defective fibrin polymerization and cross-linking, decreased activation of plasminogen, and resistance to lysis by plasmin.⁵² These abnormalities are reflected in the routine coagulation assays as prolongation of PT, APTT, fibrinogen, reptilase time, and thrombin time. The fibrinogen concentration via functional assay is low, but it is normal when measured immunologically. An important point to consider is that the abnormal fibrinogen may not be incorporated into the clot, and the soluble molecules that remain in the serum can be mistaken for fibrinogen degradation products in some assays, leading to the misdiagnosis of active fibrinolysis and thus DIC. Given the low frequency of inherited dysfibrinogenemia, routine testing in thrombophilic testing is not recommended. Dysfibrinogenemia can be seen in a variety of acquired conditions but most commonly severe liver disease.⁵²

Elevated Plasma Factor VIII Coagulant Activity

The elevated plasma FVIII coagulant (:C) level (elevated functional and antigenic levels) is considered an independent risk factor for thrombosis.⁵³ This abnormality could not be attributed to inflammation, because fewer than 10% of the patients with FVIII:C levels of greater than 150% had elevations in an acute phase reactant (such as C-reactive protein, fibrinogen, and erythrocyte sedimentation rate [ESR]).^{53,54}

Studies have demonstrated that patients with sustained FVIII greater than 150% had an increased risk of thrombosis compared with patients with FVIII less than 100%.^{53,54} One study has demonstrated that an elevated FVIII level may also be a strong thrombotic risk factor in the black population.⁵⁵ The clinical utility of routinely measuring FVIII levels in patients with thrombosis remains to be determined.

► ADVANCED CONTENT

Lipoprotein a and Thrombosis

Lipoprotein a (Lpa) represents a low-density lipoprotein (LDL)-like particle having a protein moiety apolipoprotein B-100 linked by a disulfide bridge to a glycoprotein called apolipoprotein a (Apo a).⁵⁶ Elevated levels of Lpa are under genetic control and have been recognized as an atherothrombogenic factor, but the underlying mechanisms

for this pathogenicity are not well understood.⁵⁷ There is a structural homology of Apo a with plasminogen, and laboratory studies have shown that Lpa inhibits fibrinolysis. It effectively competes with plasminogen for binding to fibrin or endothelial cells. It also binds tPA and may also stimulate the release of PAI-1 from endothelial cells. Studies show evidence of increased levels of Lpa in atherosclerosis, thrombogenesis and an association with vascular occlusive disorders.⁵⁷ However, data supporting a role in venous thromboembolism is contradictory.⁵⁸

Other Coagulant Factors Associated With Thrombosis

High levels of FXI, IX, fibrinogen, IL-8, and TAFI and low levels of plasma fibrinolytic activity or HCII are associated with venous thrombosis.⁵⁹ Assays for some of these proteins are not widely available, and the clinical utility of these measurements is currently uncertain.

Acquired Thrombotic Disorders

The two acquired thrombotic disorders are lupus anticoagulant/antiphospholipid syndrome and heparin-induced thrombocytopenia.

Lupus Anticoagulant/Antiphospholipid Syndrome

Antiphospholipid (aPL) syndrome is one of the acquired thrombotic disorders. The clinical features of aPL syndrome include vascular thrombosis and pregnancy morbidity or complications.⁶⁰ The thrombosis can be arterial or venous and tends to recur in the same site. According to the revised international classification, aPL syndrome requires the combination of at least one clinical and one laboratory criterion (Table 29-7).^{61,62} aPL syndrome is considered primary when not associated with any identified underlying disorder, and secondary when it occurs in association with another disorder. Secondary aPL syndrome is most frequently associated with systemic lupus erythematosus (SLE) and other autoimmune diseases,^{63,64} but it has occasionally been seen after exposure to certain pharmaceuticals (such as phenothiazines, quinidine, hydralazine, procainamide) and in patients with underlying malignancies. The overall prevalence of aPL syndrome is difficult to determine because of variations in laboratory techniques and diagnostic criteria. In spite of this, the prevalence of aPL syndrome in SLE patients has been examined by many researchers. The frequency of lupus anticoagulant (LA) and anticardiolipin (aCL) in nearly 2,000 SLE patients reported in the literature is 31% and 40%, respectively.⁶¹ The estimated frequency of aPL antibodies in healthy individuals is estimated to be between 1% and 5%. In SLE patients, aPL antibodies are significantly higher, around 30% to 40%.⁶⁵ Patients with SLE and aPL antibodies have a significantly higher risk of thrombotic events over time (50% to 70%), although not all patients develop thrombosis.⁶⁵ Although antibodies directed against many other antigen specificities have been reported, the major antigen targets of aPL antibodies are

TABLE 29-7 Revised Classification Criteria for the Antiphospholipid Antibody Syndrome^{75,76}

aPL syndrome is present if at least one of the clinical criteria and one of the laboratory criteria are present:

Clinical^a

- Confirmed vascular thrombosis
 - Venous
 - Arterial
 - Small Vessel
- Pregnancy morbidity, to include one of the situations described here:
 - Fetal loss at ≥ 10 weeks EGA
 - Premature delivery at ≤ 34 weeks EGA due to severe pre-eclampsia, eclampsia, or severe placental insufficiency
- Three or more unexplained consecutive fetal loss events before 10 weeks EGA.

Laboratory^{b,c}

- IgG and/or IgM aCL antibody greater than the 99th percentile for the assay (ELISA or automated systems) for the reference population per ISTH SSC guidelines
- IgG and/or IgM Anti- β_2 GPI antibody greater than the 99th percentile for the assay for the reference population per ISTH SSC guidelines.
- LA detected per ISTH SSC guidelines
- Triple aPL-positive patients are at high risk of thrombosis or aPL-related pregnancy morbidity

EGA = estimated gestational age; aCL = anticardiolipin; LA = lupus anticoagulant; β_2 GPI = β_2 -glycoprotein 1; aPL = antiphospholipid; ISTH = International Society on Thrombosis and Haemostasis; SSC = Scientific Standardization Subcommittee.

^aMust occur within 5 years of a confirmed positive test result

^{b,c}At least one of the tests must be positive on two or more separate occasions at least 12 weeks apart

β_2 GPI and prothrombin.⁶⁵ Patients who are negative for all criteria for aPL antibodies but present with APS symptoms are termed "non-criteria" aPL.⁶⁶ The autoantigen specificities of these aPL comprise various phospholipids, phospholipid-binding proteins, and coagulation factors.⁶⁶ Three aPL antibody tests are included in the APS classification criteria. Detection of IgG and IgM isotypes of anticardiolipin antibodies (aCL), anti-beta2-GPI antibodies (a β_2 GPI), and a phospholipid-based blood coagulation assay for the dysfunction caused by these antibodies is known as the lupus anticoagulant (LA) assay.^{65,66}

The term LA refers specifically to the laboratory in vitro phenomenon of prolongation of phospholipid-dependent coagulation assays such as the APTT in the absence of an attributable factor deficiency. Laboratory evidence of aPL antibodies can be in the form of either immunologically demonstrated aPL antibodies or, more frequently, LA, or both.

The presence of aPL antibodies, either demonstrated by LA or detected immunologically, is not sufficient for the diagnosis of aPL syndrome. Additional clinical features must also be present to diagnose aPL syndrome because aPL antibodies alone may not carry a similar clinical significance. An anti-phospholipid antibody profile as a biomarker for thrombophilia in SLE should help predict the risk of thrombosis.^{65,67}

Non-criteria aPL antibodies include anti-phosphatidylserine/prothrombin complex (aPS/PT), domain I specific a β_2 GPI, IgA isotype a β_2 GPI, anti-annexin V, and anti-protein S/protein C.⁶⁶ Role of anti-phosphatidylserine/prothrombin antibodies in anti-phospholipid syndrome is still a matter of debate.⁶⁸

In particular, infection-associated aPL antibodies are usually transient and are rarely associated with thrombotic complications.⁶⁸ Most aPL antibodies detected in children are of this type.⁶⁸

Mechanism of Thrombosis

Thrombosis remains the major cause of death in systemic lupus erythematosus (SLE). Antiphospholipid antibodies (aPL) are the main triggers of thrombophilia in patients with SLE, with a frequency of approximately 30% to 40%.⁶⁵ Lupus anticoagulant, anticardiolipin, and anti- β_2 -glycoprotein 1 (β_2 GPI) antibodies are included in the criteria for antiphospholipid syndrome.⁶⁵ The mechanism of thrombosis in lupus anticoagulant/antiphospholipid syndrome is not entirely clear.⁶⁹ It is likely that multiple disturbances of hemostatic mechanisms are required to result in thrombophilia.⁷⁰ One could assume that, if they were part of the etiology, they would affect phospholipid-dependent reactions in vivo and in vitro. In vitro evidence of such effects has been investigated and include inhibition of activated protein C, inhibition of AT-dependent anticoagulant mechanisms, inhibition of fibrinolysis, induction of increased soluble tissue factor, inhibition of prostacyclin secretion, promotion of platelet activation, interference with the anticoagulant properties of β_2 GPI, and displacement of annexin V from trophoblast.^{71,72} Annexin V is a protein that has potent anticoagulant properties in vivo and that may modulate thrombosis on the surfaces of cells lining placental and systemic vasculatures.⁷³ Interference with the function of annexin V may play a role in aPL syndrome in which there is recurrent pregnancy loss.^{73,74}

Laboratory's Contribution to Diagnosis

Overall, the presence of LA is a better predictor of thrombosis and pregnancy loss than the presence of high-titer anticardiolipin antibodies.^{73,74} However, some patients with aPL syndrome do not have both LA and aCL antibodies. In addition, patients who are triple-positive for aPL (i.e., LA-positive and

IgM and/or IgG aCL antibodies, and IgM and/or IgG B2GP1 antibodies) are at much higher risk for thrombotic events, as well as pregnancy morbidity.^{73,74} Therefore, the laboratory diagnosis of aPL syndrome should include both coagulation assays for LA and immunological assays for aPL antibodies. Some authors have suggested that a broad panel of aPL antibodies should be included when investigating aPL syndrome as a possible cause for recurrent fetal loss.⁷¹⁻⁷⁴

The following discussion focuses on the hematology laboratory's role in working up a suspected case of aPL syndrome: evaluation of LA. There is no single test to detect lupus anticoagulant, but screening tests, mixing tests, and confirmatory tests are usually performed.⁷⁸

As in most diagnostic algorithms, initial assays with broad sensitivity are performed and, if positive, are followed by confirmatory assays that are more specific (Table 29-8). Several guidelines have been proposed for the diagnosis of LA by the International Society of Thrombosis and Haemostasis (ISTH),⁷⁵ the British Committee for Standards in Haematology (BCSH),⁷⁶ and the Clinical and Laboratory Standards Institute (CLSI),^{75,76,77} for which the basic requirements/principles are summarized as follows:

- *Prolongation of at least one phospholipid-dependent clotting assay.* Because of the heterogeneity of these antibodies, no single screening test will detect all of them. A minimum of two "screening" assays should be available in a laboratory offering LA testing. The first analysis considered should be dRVVT (dilute Russell's viper venom time) and the APTT-based LA-sensitive assay (PTT-LA).^{75,77}
- *Evidence of inhibition of clotting is demonstrated by mixing studies (mandated by the ISTH and BCSH).*^{75,76} This involves mixing the patient's plasma and pooled normal plasma and repeating the phospholipid-dependent clotting assay in which the prolongation of clotting time was observed. If the prolongation disappears ("corrects") with mixing, this usually indicates that there is a factor deficiency. While ISTH and BCSH mandate this, CLSI does not, given the potential for false-negative reporting.^{75,77}
- *If the screening test is prolonged, evidence of phospholipid dependence is required for confirmation.* Confirmatory assays demonstrate phospholipid dependence by demonstrating a reversal of the LA effect when excess phospholipid is added to the test mixture. This can be in the form of excess phospholipids (dRVV confirm), hexagonal (II) phase phospholipids (aPTT based-confirm), and platelets or platelet vesicles (platelet neutralization test). The excess phospholipid binds and neutralizes the LA, leaving enough phospholipid to provide a surface on which coagulation reactions can proceed. A confirmatory assay that corresponds to the initial type of assay (and mixing study, if performed) that was positive should be used. Solid-phase assays for aPL antibodies, such as the aCL assay, should not be considered confirmatory for LA activity.^{78,79}
- *In addition, the absence of clinical or laboratory evidence of a specific inhibitor of any one coagulation factor may also be necessary,* because specific factor inhibitors can cause mixing studies to remain uncorrected. The clinical presentation is very important and may be informative. Patients with factor inhibitors (including those unrelated to LA, such as an acquired FVIII inhibitor) have a bleeding diathesis rather than thrombosis.⁷⁵

TABLE 29-8 Screening and Confirmatory Assays That Can Be Used in LA Testing

If This Screening Assay Is Positive, This Assay Is Recommended ...	One of These Techniques Should Be Used as a Confirmatory Test
Dilute Russell's viper venom test (dRVVT)	dRVVT confirm
APTT-based LA-sensitive assay (PTT-LA)	Hexagonal phase phospholipid test (e.g., Staclot LA) Platelet neutralization procedure Phospholipid dilutions Bovine phospholipids
Other options/historical testing	
Taipan snake venom time	Platelet neutralization procedure
Dilute prothrombin time (thromboplastin inhibition test)	Phospholipid dilutions of same
Kaolin clotting time	Platelet vesicles

Screening assays detect a prolonged clotting time. The screen procedure is suggestive of LA whenever the results are prolonged beyond the cut-off established in the laboratory. Confirmatory assays demonstrate whether or not the prolongation is decreased by phospholipids capable of neutralizing the LA antibody.^{71,72,75}

These steps can be carried out in various ways, using various discrete assays or by using integrated systems for the diagnosis of LA that incorporate an initial assay (mixing study) and a procedure that confirms phospholipid dependence into a single kit. Table 29-8 lists screening and confirmatory assays that have been used, and an approach to the diagnosis of LAs is illustrated in Figure 29-6.

In addition to the requirements for diagnosis previously described, several other elements are crucial to arriving at the correct diagnosis in cases suspicious for aPL syndrome, including:

1. Consideration of anticoagulation in the sample needs to be determined and may affect the results (i.e., false-positive results with DOACs).⁷⁹
2. Immunological assays for antiphospholipid antibody should also be done, as described in Table 29-7.
3. Testing should be repeated after a minimum of 12 weeks to demonstrate persistence of the LA. Transient aPL antibodies do not appear to be associated with the clinical complications of aPL syndrome.
4. Diagnosis of aPL syndrome should only be made in the presence of the appropriate clinical findings within 5 years of confirmed positive testing results, as described in Table 29-7.

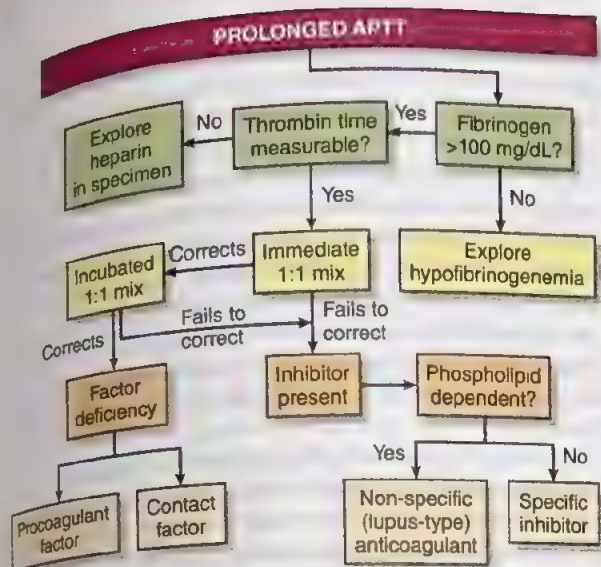


FIGURE 29-6 In the hematology laboratory, detection of LA usually begins with an elevated APTT. This figure is a flowchart that illustrates an approach to the diagnosis of LA. The shaded boxes show the typical abnormalities that can be seen when LA is present in the sample. (Courtesy of John D. Olson, MD, PhD; Department of Pathology, University of Texas Health Science Center, San Antonio, TX.)

In rare cases, catastrophic aPL syndrome (CAPS) may develop, requiring correlation with additional/alternative clinical criteria.⁷³

Algorithmic Approach to Diagnosis

In the hematology laboratory, detection of aPL antibodies usually begins with the APTT.⁷² In this discussion, we trace the laboratory diagnosis of LA as depicted in Figure 29-7. Many but not all patients with a LA will have a prolonged APTT. Some APTT assays are more sensitive (usually due to lower concentrations of phospholipid in the reagent set) to LA than others and may vary in their value as initial tests.^{72,73} Remember that prolongation of the APTT also occurs with heparin therapy, heparin contamination, insufficient volume of blood for the amount of anticoagulant in the sample tube (short draw), DIC, factor deficiencies, and specific coagulation factor inhibitors.^{75,79}

Clinical history elucidates the cause of some prolonged APTTs, and depending on the clinical situation, other assays such as the thrombin time and fibrinogen level may be employed to identify heparin contamination or DIC.⁸⁰ If the prolonged APTT is unexpected or not explained by the causes mentioned earlier, the laboratory investigation for LA is warranted, especially in a patient with a history of thrombosis or other findings indicating aPL syndrome.

CRITICAL THINKING QUESTION

29-3 What would be involved in the LA screen and confirm testing of a patient with an acquired FVIII inhibitor?

Therapy and Monitoring

Because LA can prolong coagulation tests, it may interfere with the usual tests (APTT and sometimes prothrombin time [PT]) used to monitor anticoagulation. When the baseline APTT is prolonged in a patient with LA, then a heparin (anti-Xa) assay is recommended to monitor unfractionated heparin therapy. Warfarin therapy is monitored by the PT assay, with an international normalized ratio (INR) of 2 to 3 being a typical therapeutic window.⁸¹ However, this is a complex issue for two reasons: (1) there is controversy regarding the optimal degree of anticoagulation and (2) some thromboplastins are more sensitive to the effects of LAs than others.⁸² Monitoring therapy with a PT using one of these thromboplastins can be misleading even if the baseline INR of the patient with LA is within the reference range. During anticoagulation, the INR may be prolonged by the LA and appear to be within therapeutic range, although the patient is actually insufficiently anticoagulated.

The effect of LA on the PT is not predictable, creating problems when determining the appropriate INR to use for oral anticoagulation. Because of the variable effect, it would be prudent to determine the therapeutic INR for each patient with LA who receives oral anticoagulant therapy. The goal of therapy is to reduce the functional level of vitamin K-dependent factors in the plasma; therefore, an assay of one of the factors to confirm the therapeutic effect

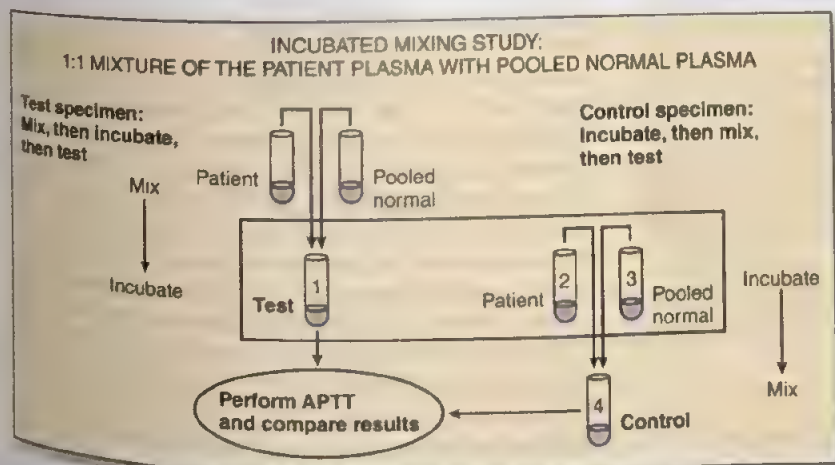


FIGURE 29-7 Incubated mixing study can be performed as shown in this figure. For details, refer to the accompanying text. (Courtesy of John D. Olson, MD, PhD; Department of Pathology, University of Texas Health Science Center, San Antonio, TX.)

should be done after the patient is receiving a stable dose of anticoagulant. The assay of FX is the most frequently used, with a target of 20% to 25% activity.⁷⁹ The LA can also affect clot-based assays, so the assay for FX needs to be an assay that uses a chromogenic substrate in a reaction that is not phospholipid-dependent. Once the FX is confirmed to be in the therapeutic interval, the corresponding INR can be used for monitoring, if the INR is in a sensitive range (i.e., less than 6.0).⁷⁹ For the rare patient whose LA has a profound effect on the INR, precluding its use, periodic assay of FX may be necessary.

Heparin-Induced Thrombocytopenia

Heparin-induced thrombocytopenia (HIT) is another antibody-mediated cause of venous and arterial thrombosis and is a life-threatening disorder that follows exposure to unfractionated heparin (less commonly to low molecular weight heparin).⁸¹ Studies have shown that between 1% and 5% of hospital patients exposed to heparin for 1 to 2 weeks develop HIT.⁸² Of patients diagnosed with HIT, approximately one-third will develop overt thrombosis, and of these, about one-third will suffer amputation or death.⁸³ Early recognition and appropriate treatment may reduce these numbers.^{81,83-85}

Heparin is a widely used anticoagulant that can be administered intravenously (IV) or subcutaneously (SC) to prevent thrombosis in high-risk patients and to limit progression of established thrombosis. It is also used as a flush to keep IV lines open. Heparin is often used to prevent clotting in extracorporeal circulation such as that in heart-lung bypass machines, where it is infused or even present as the anticoagulant coating within the tubing system.

HIT is characterized by an unexplained decrease in platelet count, occurring 5 or more days after the initiation of heparin therapy.⁸¹⁻⁸³ This lag is consistent with an immune response related to heparin administration unless the patient has been previously exposed to heparin.

Nomenclature seen in the literature regarding this syndrome can be confusing. Over the years, various names have been used for this syndrome, such as heparin-induced thrombocytopenia with thrombosis syndrome (HITS) and HIT type II. These terms have been used to distinguish immune-mediated HIT from the mild, non-immune-mediated thrombocytopenia, which may occur within the first few days of heparin administration.^{81,82} This nonimmune (also called HIT type I) thrombocytopenia resolves spontaneously and does not increase the risk for thrombosis. In addition, delayed HIT has also been described, in which the platelet count starts to decrease *more than 5 days after stopping heparin*.⁸² The proposed mechanism for this is related to high titer antibodies capable of both heparin-dependent and heparin-independent (i.e., capable of recognizing non-heparin-bound PF4) platelet activation.⁸⁶ For the rest of this chapter, when the term *HIT* is used, it signifies the immune-mediated disorder in which there is a risk of thrombosis.⁸⁷

Clinical Manifestations

Although HIT may present with simultaneous thrombocytopenia and thrombosis, or sometimes with thrombosis preceding

thrombocytopenia, the first manifestation of HIT is usually an unexplained decrease in platelet count of 30% to 50% or to less than $100 \times 10^9/L$, occurring 5 or more days (usually 5 to 8 days) after the initiation of heparin therapy.⁸¹⁻⁸³ The platelet count rarely decreases to $15 \times 10^9/L$ or less.^{81,83}

HIT can cause both venous and arterial thrombosis, but venous thrombosis occurs about four times more often. DVT, PE, lower limb arterial thrombosis, and coronary arterial thromboses may occur.⁸⁷ Other sequelae can also be seen. Thromboses may be multiple. The occurrence of multiple arterial thromboses is sometimes referred to as "white clot syndrome," because the thrombi formed in high-flow vessels have a high platelet and fibrin content and relatively few red blood cells.⁸⁷

Accurate diagnosis of HIT requires a high degree of suspicion on the part of the physician caring for the heparin-exposed patient. Recommendations for monitoring platelet counts vary, but it is prudent to check a baseline platelet count at the initiation of heparin therapy and to repeat platelet counts at intervals of several days. Because of the timing of the onset of HIT, particular vigilance around day 4 after initiation of heparin (day 0 being the first day of heparin administration) and for 10 days thereafter is particularly crucial. Patients who have had previous exposure to heparin may have an anamnestic response and develop HIT rapidly after repeat heparin exposure. Many other causes for a decreased platelet count should be considered before rendering a diagnosis of HIT. Among these are fever, DIC, splenomegaly, and medications (other than heparin). Of note, in spite of the thrombocytopenia, HIT patients rarely have a bleeding diathesis.

Although several clinical probability scores have been studied, the most commonly used and recommended is the "4T score."⁸⁸ This clinical score has such a high negative predictive value that many organizations do not recommend testing with a low 4T score (0-3) to further exclude HIT.^{82,88}

If suspicion of HIT is high (4T score of 4 or higher), all sources of heparin exposure should be discontinued immediately.^{82,88} Continued heparin exposure greatly increases the risk of thrombosis and LMWH should not be administered, as there is a cross-reactivity rate of 93%.⁸² Assays are available to assist in the diagnosis of HIT, but discontinuation of heparin should not wait for these results. Alternative anticoagulant therapy—direct thrombin inhibitors (hirudin analogs, argatroban, fondaparinux, or DOACs) should be considered because of the high risk of thrombosis even after heparin is discontinued.^{82,83,87} Platelet count often rises rapidly after the discontinuation of heparin. The return of the platelet count to normal within 5 to 7 days of discontinuation of heparin is consistent with a diagnosis of HIT, although some have been observed to take up to a month to recover completely. As with most immune reactions, heparin antibodies may remain in the plasma for extended periods of time. However, testing should occur within 6 weeks of a thrombocytopenic event.⁸²

The risk of HIT appears to be greater in patients exposed to large amounts of heparin, such as when systemic anticoagulation is required. However, patients with exposures to very small amounts of heparin, such as that used to keep IV lines from clotting when not in use, have also developed HIT.⁸²

Mechanism
When heparin complexes with PF4, it forms the antigen usually responsible for initiating HIT (Fig. 29-8).⁸⁶ PF4 is present in the α granules of platelets and is released when heparin or other agonists activate them. Antibodies to these heparin-PF4 (H-PF4) complexes form in almost all HIT patients.^{85,86} IgM, IgA, and IgG specific for H-PF4 antigen have all been detected, although IgG is most common and pathogenic.^{85,86} When the H-PF4-antibody immune complex is bound by platelet Fc γ RIIa receptors (a subtype of receptor found on platelets that binds the constant portion of immunoglobulin molecules), two things may happen. First, splenic macrophages may remove the platelets, that are now coated with Ig attached to the platelet FC receptors; second, the attachment of the immune complex to the FC receptor can result in platelet activation. The activated platelets release substances that attract and activate more platelets. Levels of thrombin increase, which along with platelet-derived microparticles can ultimately result in thrombosis.⁸⁷

The fact that many patients form these antibodies without developing HIT suggests that other factors are required for the development of HIT besides H-PF4 antibodies.⁸⁷ One fascinating and controversial possibility is that the His-131 polymorphism in the gene for the platelet Fc γ RIIa receptor may increase susceptibility to HIT.^{85,86}

Unfractionated heparin (UH), the most commonly used heparin, is the major medication associated with HIT,

occurring less often with porcine UH than with bovine UH. Low molecular weight heparin can also cause HIT, although at a much lower rate than UH, which is part of the reason some institutions have replaced UH with LMWH in as many situations as possible.^{85,88}

Laboratory Diagnosis

Two types of assays are commonly used to assist in the diagnosis of HIT, immunological assays and functional assays. Types of immunological assays include particle gel immunoassays (e.g., PaGIA), ELISAs, latex-particle-enhanced assays, as well as chemiluminescent assay.^{89,90} These assays use the H-PF4 complex as the target antigen to detect HIT-type immunoglobulin (Ig) in the patient's serum. Initially these assays were primarily polyspecific in isotype (e.g., IgG, IgA, and IgM antibodies) with a high sensitivity but relatively low specificity for pathogenic antibodies.⁸⁹ However, current guidelines support screening with IgG-specific versus polyspecific assays.^{89,90} With the appropriate clinical information, these assays have an extremely high negative predictive value. However, confirmatory testing is necessary with functional assays, as the false-positive rate is still high.

Functional assays may look for platelet aggregation or may be a variation on a platelet aggregation method that detects products of the platelet release reaction, such as serotonin or adenosine triphosphate (ATP). These assays use the patient's

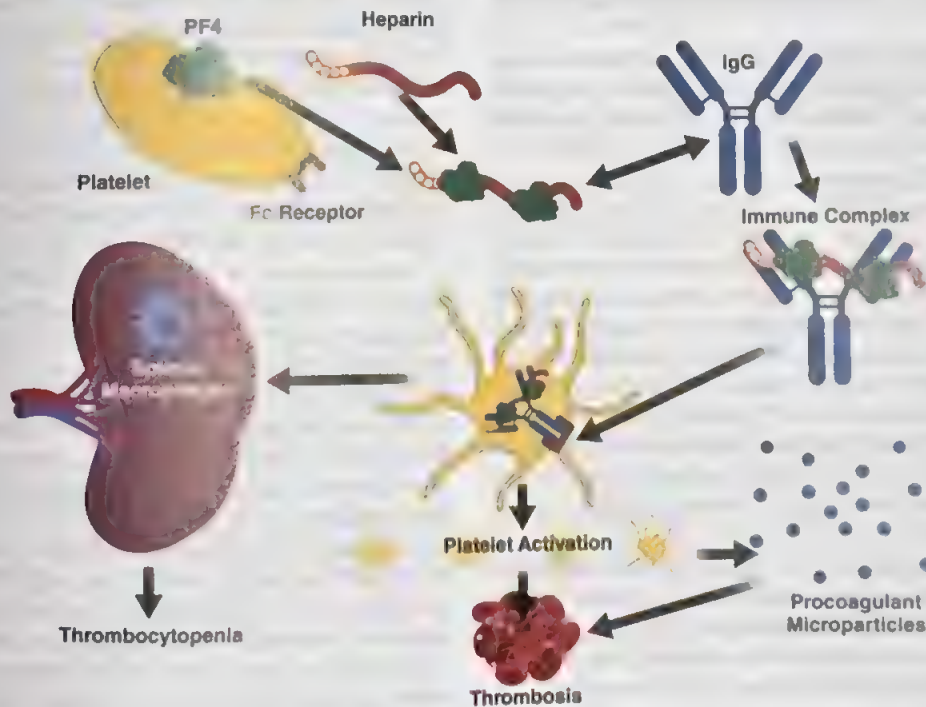


FIGURE 29-8 Pathophysiological mechanism of heparin-induced thrombocytopenia (HIT). Platelet factor 4 (PF4) is a chemokine secreted from the alpha-granules of platelets, released as tetramers. They bind to heparin and other proteoglycans and inactivate them. The binding of heparin to PF4 exposes new antigen sites and hence the formation of new (IgG) antibodies. Platelet FC receptors bind the antibody-heparin-PF4, which contribute to thrombosis. Thrombocytopenia occurs by two mechanisms: removal of platelets with bound IgG by splenic macrophages and platelet consumption caused by thrombus formation. This figure was created using the website <https://app.biorender.com> (accessed on 12 July 2021). Adapted from Acanfora D, Acanfora C, Ciccone M, Scicchitano P, Bortone A, Uguccioni M, et al. The Cross-Talk between Thrombosis and Inflammatory Storm in Acute and Long COVID-19: Therapeutic Targets and Clinical Cases. *Viruses*. 2021;13(1904):1-14. Reproduced with permission under the guidelines of the Creative Commons Attribution 4.0 International Public License ("Public License") via <https://creativecommons.org/licenses/by/4.0/>.

serum, heparin, and donor platelets. Either bovine or porcine heparin may be used in the assays. There is no need for the laboratory to determine whether the patient has received bovine or porcine heparin. It is important to use platelets from donors whose platelets are known to be reactive to HIT sera. It is unknown why some donors' platelets are reactive and others are not. Occasional combinations of known HIT sera and known reactive HIT-reactive platelets do not aggregate. Therefore, it has been suggested that platelets from two donors whose platelets are known to react to HIT sera should be used. If platelet aggregation occurs or if there is evidence of the release reaction only at a low concentration of heparin (0.1 U/mL), this is evidence that the patient's serum contains antibodies that activate and aggregate platelets in the presence of a therapeutic concentration of heparin.⁹⁰

In any of these functional assays, it is important to use appropriate controls to exclude the presence of non-heparin-induced aggregation. In addition to using a control that lacks heparin, the use of a control containing a high concentration of heparin (100 U/mL) is included.⁹⁰ When HIT serum is tested using a high concentration of heparin, aggregation should not occur.⁹¹ It is reasonable that this might be caused by antigen (heparin) excess, but nonspecific inhibition of platelet aggregation in response to other agonists (collagen, adenosine diphosphate [ADP], and epinephrine) has also been observed. In any case, aggregation of platelets at both low and high concentrations of heparin is considered to be evidence that the aggregation is caused by a mechanism other than that of HIT.^{91,92}

One of the functional assays is the serotonin release assay (SRA).^{91,92} It is currently the primary comparison procedure because most of the available data on clinical outcomes have been gathered in studies using this assay. Donor platelets are incubated with [¹⁴C]serotonin, to allow its uptake into their dense bodies. These platelets are then washed, removing free [¹⁴C]serotonin that has not become incorporated. They are then exposed to the serum of the patient to which is added a therapeutic concentration of heparin. If the patient's serum contains H-PF4 antibodies capable of activating platelets, then the radioactively labeled serotonin will be released from the dense granules. The test specimen is then centrifuged and the supernatant tested for ¹⁴C activity. This assay has high sensitivity (88% to 100%) and high specificity (89% to 100%).^{91,92} However, it is time-consuming and requires the use of radioisotopes, limiting its availability primarily to reference laboratories.

Flow cytometric methods for diagnosing HIT have also been developed. They employ fluorescent antibodies against platelet glycoproteins and forward angle light scatter (indicating cell size) to detect platelet microparticles. These microparticles are generated when donor platelets are exposed to patient sera, causing platelet aggregation in the presence of therapeutic concentrations of heparin. These methods seem promising in their ease of performance and have been observed to correlate well with the SRA.

The clinical diagnosis of HIT often remains unconfirmed by any of these laboratory techniques. Sensitivities and specificities reported for these assays vary but overall are suboptimal.

No single technique is reliable enough to be considered a gold standard for the diagnosis of HIT.^{91,92} Sensitivity and specificity can be improved by using more than one methodology to test for heparin-associated antibodies. In cases suspicious for HIT, repetition of the assays every few days may provide confirmation. Patients must be removed from heparin for up to 4 hours before a sample is collected. The presence of heparin in the sample may cause a false-negative result, especially in functional assays.⁹² Clinical decision-making should be based predominantly on clinical impression. However, laboratory evidence of HIT is often helpful in supporting the decision to discontinue heparin and use an alternative anticoagulant.

Therapy and Monitoring

The effective management of patients with HIT is to prevent thrombosis by limiting platelet activation and thrombin generation.⁸² Stopping all heparin exposure is the most important step in preventing or limiting thrombosis in patients with HIT.⁸⁷ However, even after cessation of heparin, the patient still has a risk of approximately one in three for developing thrombosis. LMWH is also not recommended for the treatment of HIT.⁸⁷ Although it is less likely to induce HIT once a patient has a heparin-induced antibody, exposure to LMWH carries a risk of thrombosis because of high cross-reactivity with PF4-heparin antibodies.⁹² However, fondaparinux, has only been associated with rare case reports of HIT.⁹³ Clinical correlation is necessary at this point. If a diagnosis of HIT is made without known thrombosis, a venous Doppler of all four extremities to evaluate for subclinical thrombosis is recommended.⁸⁷ HIT is considered a provoked thrombotic event with the duration of anticoagulation based on the presence or absence of thrombosis. Patients with HIT and thrombosis should receive at least 3 months of anticoagulation from the time the platelet count normalizes.⁸⁷ Patients with HIT without thrombosis should still be anticoagulated prophylactically, but for a shorter period of time, usually 4 to 6 weeks.⁹³

Anticoagulation in HIT patients is complex with lack of significant data of efficacy in many cases, but definitively exclude unfractionated heparin and LMWH and include non-heparin anticoagulants (such as argatroban, bivalirudin, danaparoid, fondaparinux, direct oral anticoagulants).⁷⁶ Currently *approved* agents are all continuous infusions, requiring hospitalization for monitoring (e.g., argatroban). However, several newer anticoagulants, including fondaparinux, have been employed with great success.^{93,94} It is unlikely for large clinical trials of alternative anticoagulation (including DOACs) of HIT to be successful in recruitment and accrual, given the rarity of this disorder, suggesting off-label use of these anticoagulants will be continuing and expanding.⁹⁴

Importantly, due to the risk of venous limb gangrene (distal ischemic necrosis that is present despite palpable or Doppler-identifiable arterial pulses), the oral anticoagulant warfarin sodium should not be used in a patient with HIT unless the patient is also adequately anticoagulated by another non-heparin anticoagulant for the first few days.⁹⁵ The early warfarin-induced reduction of functioning PC in the presence of increased thrombin generation seen in HIT puts the patient at very high risk for this and other thrombotic complications.⁹⁵

Other Acquired Conditions Associated With Thrombosis

Other acquired conditions associated with thrombosis include:

- Pregnancy and the use of oral contraceptives
- Nephrotic syndrome
- Medication uses
- Cancers and other conditions
- Major trauma

Thrombosis With Pregnancy and Use of Oral Contraceptives

There is a six-fold increased risk of thromboembolism during pregnancy.^{96,97} Thrombosis during pregnancy is attributable to the following conditions:^{96,97}

- Venous stasis in the lower extremities, caused by the gravid uterus⁹⁸
- Trauma to pelvic veins during delivery
- The placenta is rich in tissue factor and also releases plasminogen activator-2, which reduces fibrinolytic activity
- Increased levels of most coagulation proteins (fibrinogen can be increased to 600 mg/dL)
- A significant decline in plasma levels of free and total PS
- Elevated D-dimer levels during the third trimester

Although PC and AT levels remain normal during pregnancy, both are decreased in preeclampsia.

The risk of venous or arterial thrombosis secondary to oral contraceptives is related to estrogen dose. Most oral contraceptives prescribed in the United States contain low-dose estrogen. However, even the use of low-dose estrogen contraceptives confers a four-fold increased risk of thrombosis compared with nonusers. The risk may become significant if the woman also has an inherited risk factor for thrombophilia. The exact mechanism of the prothrombotic state is not well understood, but it is thought that these medications may cause an acquired APC-R-like state.⁹⁹

Thrombosis and Nephrotic Syndrome

Nephrotic syndrome is characterized by heavy proteinuria (more than 3.5 g/day), hypoalbuminemia, severe edema, and hyperlipidemia, and thrombosis of both venous and arterial systems may be seen.¹⁰⁰ The renal vein is the most common site involved and is present in approximately one-third of patients.¹⁰⁰ The incidence of thrombosis varies and correlates with the severity of the disease. Depending on the complex interaction between hepatic protein biosynthesis and the severity of renal disease, the following changes of coagulation parameters can be seen: AT levels are often decreased; PC and S are increased; and factors V, VII, VIII, X, and XIII are increased, whereas factors XI and XII are decreased. In addition, platelet hyperactivity can also be seen in these patients.¹⁰⁰

Cancer-Associated Thrombosis (CAT)

Patients with cancer are at high risk of both arterial and venous thrombotic events compared with the general population.¹⁰¹ The estimated incident rate of venous thromboembolism (VTE) in patients with cancer is approximately 4% to 20% and the incident rate of arterial thromboembolism is estimated

to be 2% to 5%.¹⁰² Risk factors that lead to CAT include cancer type, chemotherapy, radiotherapy, hormonal therapy, antiangiogenesis therapy, surgery, or supportive therapy with hematopoietic growth factors.¹⁰¹

The increased risk of thrombotic events is attributed to the site and stage of cancer.¹⁰¹ The risk of thrombosis is enhanced in several treatment-related factors: hospitalization, surgery, central venous catheters, radiation, and anticancer agents.¹⁰²

Greater than 100 chemotherapy and targeted therapy drugs have been approved for the treatment of a variety of cancers. Cancer or malignancy induces a thrombophilic state by increasing the risk of venous stasis. In addition, a hypercoagulable state is caused by an imbalance of pro- and anti-thrombotic factors and endothelial injury.¹⁰³ "Anticancer agents may damage the endothelium, decrease anticoagulants, or increase procoagulants leading to activation of coagulation or platelets."¹⁰⁴ "Myocardial infarctions and cerebrovascular accidents (CVAs) account for the highest proportion of thrombosis-associated deaths in the United States."¹⁰⁵

Myeloproliferative disorders predispose patients to venous or arterial thrombosis, especially when the platelet count and hematocrit are not controlled by therapy.¹⁰⁶ Increased blood viscosity and activation of platelets may be the cause for thrombosis.¹⁰⁷ In patients with paroxysmal nocturnal hemoglobinuria (PNH), there is a predilection for thrombosis in intra-abdominal and cerebral vessels.¹⁰⁸ A diagnosis of PNH should be suspected in patients with pancytopenia, elevated reticulocyte count, low iron studies, and a negative family history of thrombosis.¹⁰⁹

The following cancers are associated with a high risk of thrombosis: lung, esophagus, stomach, bowel, pancreas, brain, and hematological malignancies.¹¹⁰

Diagnostic Approach and Issues in Laboratory Testing

The purpose of detecting hypercoagulable states is to predict and prevent thromboembolic disease and its complications, identify risk factors, modify treatment regimens, and discover underlying diseases that might be amenable to treatment¹¹¹ (Boxes 29-1 to 29-5). The diagnostic evaluation of patients with thrombotic diathesis initially involves consideration of two factors: (1) whether the disorder is inherited or acquired and (2) whether the thrombosis involves primarily the veins or arteries¹¹² (see Table 29-9). For example, a 35-year-old person with recurrent DVT is most likely to have an inherited defect of one or more of the anticoagulant proteins.¹¹² On the other hand, the hypercoagulable state in a 70-year-old bedridden person is most likely to be acquired. Therefore, knowledge of the coagulant, anticoagulant, and fibrinolytic systems; complete history and physical examination; and ethnic background (factor V Leiden is very rare in African Americans and Asians) are necessary.¹¹³ The basic, but very important, step that needs to be considered before ordering assays for inherited disorders is identification of the risk factors.¹¹⁴

Specific laboratory tests of interest in patients with thrombophilia have been addressed in other sections of this chapter. There are, however, some issues that deserve consideration when approaching the laboratory evaluation of a

BOX 29-1 Differential Diagnosis of Hypercoagulable States**Hereditary Conditions**

- Factor V Leiden mutation
- Prothrombin gene mutation
- AT deficiency
- Protein C deficiency
- Protein S deficiency
- Heparin cofactor II deficiency
- TFPI deficiency
- Elevated lipoprotein a
- Homocysteinemia
- Factor XII deficiency
- Elevated factor VIII levels
- Plasminogen deficiency
- Increased plasminogen activator inhibitor

Acquired Conditions

- Antiphospholipid antibodies
- Heparin-induced thrombocytopenia
- Malignancy
- Autoimmune disorders
- Nephrotic syndrome
- Myeloproliferative disorders/PNH
- Behçet's syndrome
- Microangiopathic hemolytic anemia (DIC, TTP, HUS)

Note: The list is not exhaustive; other, rare causes of thrombophilia are not included.

BOX 29-2 Risk Factors for Venous Thromboembolism

- Pregnancy
- Oral contraceptives
- Obesity
- Surgery
- Trauma
- General anesthesia
- Malignancy
- Immobility
- Varicose veins
- Infection
- Congestive heart failure
- Advancing age
- Infusion of prothrombin complex concentrates
- Diabetes mellitus and hyperlipidemia
- Treatment related:
 - L-Asparaginase
 - Mitomycin

patient who has suffered from thrombosis. They are summarized in Box 29-6 and addressed briefly later.

Complete History and Physical Examination

A complete personal, family, and drug history is extremely important in evaluating patients with acute, recurrent, or remote thrombosis.¹¹⁴ The patient should be carefully questioned about

BOX 29-3 Evaluation of a Patient With Suspected Thrombophilia (Hereditary/Acquired)

1. Age at onset
2. Any underlying associated risk factors
3. Number of events (initial or recurrent)
4. Site of thrombosis
5. Any documented evidence of pulmonary embolism or DVT
6. Whether recurrent thromboembolism occurred despite therapeutic anticoagulation
7. Any history of recurrent fetal loss, MI, or stroke
8. Any medications that can lead to thrombosis (oral contraceptives, chemotherapy)
9. Family history of venous thromboembolism

BOX 29-4 Suggested Evaluation Criteria for Inherited Thrombophilia

1. Thrombosis at any age especially in younger patients*
2. Recurrent thrombosis
3. Significant family history of thrombosis
4. Thrombosis at unusual sites (other than deep veins of legs)

*Studies have demonstrated that the yield in detecting inherited risk factors for thrombophilia in the aged occurs at the same rate and would have the same value in guiding clinical management.

BOX 29-5 Testing for Inherited Thrombophilia**Plasma Coagulation Screening**

- PT
- APTT
- Thrombin and reptilase times

Anticoagulant System

- APC resistance assay (APTT-based assay)
- Protein C (functional and antigenic)
- Protein S (functional and antigenic)
- AT (functional and antigenic)

Fibrinolytic System

- Fibrinogen (functional and antigenic)
- Factor XII activity
- Plasminogen (functional)

Genetic Tests

- Factor V Leiden R506Q variant in the factor V gene
- Prothrombin gene mutation (nucleotide G20210A)
- *MTHFR* gene mutation

Additional Tests

- Homocysteine level
- Lipoprotein a level
- Factor VIII activity

TABLE 29-9 Coagulation Defects and Sites of Thrombosis

Defects	Venous	Arterial
Factor V Leiden*	++	-
Prothrombin gene mutation	++	?
PC deficiency**	++	-
PS deficiency	++	-
AT deficiency	++	-
Hyperhomocysteinemia	+	++
Lipoprotein a	-	++
Antiphospholipid syndrome	++	++
Heparin-induced thrombocytopenia	++	++

*Women who smoke and take oral contraceptives have an increased risk of myocardial infarction.

**A slight increased risk of stroke has been reported.

BOX 29-6 Issues in Laboratory Testing in Patients With Thrombosis

- Ensure a complete history and physical examination are obtained.
- Careful selection of tests during the acute event.
- Consider conditions that can interfere with test results.
- Test in the appropriate clinical setting.
- Use functional assays when possible.
- In arterial thrombosis, consider the additional evaluation of hyperhomocysteinemia and lipoprotein a.
- Repeat testing before diagnosis.
- Ensure that the yield in performing testing is high, making evaluation worthwhile.

all the possible risk factors for thrombosis. A significant family history of thrombosis strongly suggests the possibility of inherited thrombophilia. If the patient is female, history of oral contraceptive use and obstetric history is important. Presence of constitutional symptoms, hemoptysis, melena, or hematuria may suggest underlying occult malignancy.¹¹⁴ Recurrent thrombosis despite anticoagulation therapy may suggest underlying malignancy. Atherosclerotic vascular disease and renal disease (nephrotic syndrome) can lead to arterial or venous thromboembolism.¹¹⁵

Ethnic background is also important in evaluating patients with inherited thrombophilia, as certain diseases are more prevalent in populations, which may direct additional and/or repeat testing. Physical examination should be specifically directed to examining vascular system, skin, extremities, heart, chest, and abdomen.¹¹⁴

D-Dimer Assay in the Diagnosis of Thromboembolism

Given the nonspecific symptoms associated with vascular thromboemboli, a common clinical question is whether a potentially dangerous clot is present. The risk of fatality or

morbidity requires the use of anticoagulation if an aberrant hypercoagulable state is present, and these medications are not without risk and indeed can have a great effect on lifestyle (such as with contact sport athletes). Establishing a diagnosis of venous thromboembolism is well studied and is typically assessed using a combination of clinical history and symptoms along with clinical testing and various vascular analysis techniques.¹¹⁶

D-Dimers are the specific cross-linked fibrin breakdown products formed in the dissolution phase of clot formation, and therefore elevated levels are almost always seen in patients with VTE.¹¹⁶ A negative D-dimer test result has a negative predictive value of approximately 90%, which is useful to exclude VTE.¹¹⁶ However, the power of the negative predictive value is reduced in patients with a high prevalence of VTE (such as in malignancy or ICU patients).¹¹⁶ In these patient populations, a high index of suspicion warrants further investigation. The addition of the more specific impedance plethysmography or venous compression ultrasound yields a more accurate diagnosis. On the other hand, elevated D-dimer levels are insufficient to establish the diagnosis of VTE, as elevated levels can be seen in hospitalized patients, infection, inflammation, surgery, pregnancy, as well as other conditions.¹¹⁶

A confusing aspect of the D-dimer assay is the use of many different concentration units in which the result is reported, which makes interlaboratory comparisons difficult. D-dimers are reported as either fibrinogen equivalent units (FEU) or D-dimer units (DDU) in ng/mL, mg/L, g/mL, or g/L.¹¹⁶ Numerous assays now provide cut-offs for the exclusion of VTE, but the ideal approach would be that every hospital and lab should establish their own cut-off values to rule out VTE.¹¹⁷ Because the threshold values used in the literature may differ depending on the methodology and reporting units used, the units can be converted based on the manufacture's recommendation.¹¹⁶ The following points regarding D-dimer are important to understand in the initial diagnosis of a thromboembolic episode:

1. D-dimer testing can be a sensitive test to screen for thromboembolic disease, but is very nonspecific, so a negative test is much more helpful than a positive result.¹¹⁷
2. ELISA testing methods have the best sensitivities (95%), with a negative predictive value of 90%.
3. In patient populations where the likelihood of thromboembolism is high, D-dimer alone may not be sufficient to exclude a clotting episode.¹¹¹⁻¹¹⁷

Testing During the Acute Event

Thrombotic events, whether on the arterial or venous side of the circulation, are capable of consuming naturally occurring coagulation inhibitors (PC, PS, AT) that are of interest for testing. In addition, a number of these components are either positive or negative acute phase reactants. Anticoagulant therapy also affects these concentrations variably. For these reasons, testing during the acute event and during anticoagulant therapy will produce both false-positive and false-negative results.¹¹⁷

Conditions That Can Interfere With Test Results

Several physiological and pathological states, as well as medications, can affect plasma levels of PC, PS, and AT. One of the most common problems is the interpretation of these assays under inappropriate conditions. Levels of PC, PS, and AT are physiologically low in newborns, during pregnancy (PC may be high), and in the early postpartum states. Pathological decreases in levels of PC, PS, and AT can be seen in post-thrombotic states, the postoperative states, severe liver disease, and DIC. In nephrotic syndrome, PS and AT levels are decreased while PC levels may be increased.¹¹⁷

PC and PS are vitamin K-dependent proteins, and both functional and antigenic levels will be reduced during oral anticoagulation therapy. Warfarin has on rare occasion elevated AT levels in patients with a hereditary defect of this coagulation inhibitor. Heparin therapy may falsely lower AT activity. Heparin therapy will not affect plasma levels of PC and PS but will interfere with APTT-based activity assays. For these reasons, it is a good practice to test for these deficiency states at least 2 weeks after completing the initial 3- to 6-month course of oral anticoagulant therapy (PC and PS assays can be performed as the patient is anticoagulated with heparin).¹¹⁸ Deficiency states for PC, PS, and AT may be excluded if the levels for these proteins are normal during the acute thrombotic episode. But finding a low level of these proteins during this period requires confirmation by repeat testing after anticoagulation is discontinued and sufficient time has elapsed for natural concentrations of factors to equilibrate (approximately 2 to 4 weeks). Investigation of first-degree relatives can be useful to document the inherited nature of a deficiency.¹¹⁹

Because most patients hospitalized for thrombosis are in the acute postthrombotic state and are receiving anticoagulant therapy or have illnesses that may interfere with testing, the tests for PC, PS, and AT levels are generally better suited to the outpatient setting and have a limited role in the diagnosis of thrombophilia in hospitalized patients.¹²⁰

Genetic testing, including FVL mutation, prothrombin gene mutation, and *MTHFR* mutation, are not affected by physiological or pathological states and anticoagulation therapy. Therefore, though not typically recommended, the genetic tests can be performed in these settings.

Testing in the Appropriate Clinical Setting

The development of a thrombus, regardless of the clinical setting, generally involves the accumulation of more than one inherited or acquired risk factor.^{119,120} Venous thrombosis in almost any clinical setting merits a comprehensive evaluation. For some time, it was thought that evaluation for inherited thrombophilia should be limited to the "young" patient with thrombosis. Studies have demonstrated that the yield in detecting inherited risk factors for thrombophilia in the aged occurs at the same rate and would have the same value in guiding clinical management.¹¹⁹

Functional Assays

Many of the molecules of interest in thrombophilia can be assayed using functional or antigenic assays. Whenever quality functional assays are available, they should be the first assays performed. In general, if the assay is within the

reference range, further testing is not indicated. In contrast, if the functional assay is below the reference range, evaluation by antigenic assay is needed to determine whether a normal amount of abnormally functioning molecule is being produced.

Testing Considerations

The usefulness of testing for thrombophilia in patients with arterial disease, particularly MI and stroke, continues to be discussed in the literature. A consensus has not evolved regarding the usefulness of laboratory testing in these settings. Risks in relation to hyperhomocysteinemia and Lp(a) have been well demonstrated. LA and HIT can cause either venous or arterial thrombosis. The absence of exposure to heparin or related compounds eliminates the need to consider HIT.¹

Virtually all of the assays involved in the evaluation of thrombophilia are influenced by a variety of factors in both the preanalytic and analytic phases.¹²¹ Because of this, false-positive results are a common problem. Therefore, positive tests should be confirmed before the patient is labeled as having a deficiency state.¹²² Molecular/genetic testing is the exception.

These assays are not indicated as a routine preoperative evaluation if the aim is to detect the risk of thrombosis in the absence of a known personal or family history of thrombosis. Also, there is no valid indication for these tests in an elderly patient who has a risk factor for venous thromboembolism. Patients who present with thrombosis and who are thoroughly evaluated will have more than a 50% probability of having a thrombophilic risk factor identified.¹²³ The usefulness of determining thrombophilic risk factors continues to evolve. It is very likely that, as clinical studies evolve, treatment strategies will also evolve that will be directed by one or more of the thrombophilic risk factors.¹²³

Anticoagulant Therapy

Patients with venous thrombosis are treated with therapeutic doses of anticoagulation. This is done to prevent propagation of the thrombus, to reduce the risk of embolus, and to allow for natural resolution. In addition, patients in a clinical setting (i.e., perioperative) with a high risk of thrombosis are treated with low (prophylactic) doses of anticoagulants to prevent thrombus formation.¹²⁴⁻¹²⁶

Unfractionated Heparin Therapy

Heparin is an unbranched polysaccharide that is heavily sulfated, making it anionic. Commercial preparations are made by extracting these molecules from bovine lungs or porcine intestines. Therefore, the heparins that are extracted are variable in size (4 to 35 kD, averaging 12 to 13 kD).¹²⁷ The nature and degree of sulfation and the size of the molecule influence the biological activity.

The anticoagulant activity of heparin is to function as a cofactor. When heparin is added to purified activated coagulation factors (in the absence of AT), no anticoagulation occurs. Heparin acts by accelerating the rate at which AT is capable of binding to the activated serine protease, irreversibly inhibiting its activity.¹²⁷ In the absence of heparin, neutralization of thrombin or factor Xa will occur in a time frame of approximately 15 minutes. In the

presence of heparin, the same reaction occurs more rapidly than is possible to measure.

Administration and Monitoring

Unfractionated heparin must be administered parenterally, most frequently intravenously but it can also be used subcutaneously.^{126,127} Clearance of heparin is primarily cellular (reticuloendothelial cells) and renal filtration. At usual doses, the clearance is by the reticuloendothelial cells and provides an average half-life of approximately 60 to 90 minutes.¹²⁷ At higher doses, renal filtration becomes a factor when the reticuloendothelial system is saturated. This might explain the varying half-life of heparin with increasing dose.

The dose of heparin is determined by the weight of the patient. It is given as a bolus dose followed by continuous IV infusion. Owing to the length of the half-life of the drug, monitoring should not occur until equilibrium is reached at about 6 hours after beginning or changing therapy.¹²⁶ Once within the therapeutic interval, daily monitoring is sufficient.

When used for therapeutic purposes, heparin has a very narrow therapeutic interval. The goal, of course, is to prevent thrombosis without causing hemorrhage. If the drug is given in inadequate doses and serum concentrations are below effective levels, the consequences can be thrombosis and embolism, causing significant morbidity and even mortality. When concentrations are elevated, the anticoagulant properties will cause hemorrhage. It is the obligation of the laboratory to inform clinicians of the method that the laboratory recommends for monitoring heparin and the therapeutic interval.

Heparin therapy can be monitored by one of two strategies.¹²⁸ The first involves monitoring the effect of heparin on the patient's coagulation system, most frequently via the APTT. The second is the target concentration strategy in which the concentration of heparin in the blood is determined. Both of these strategies have been demonstrated to be effective in monitoring populations of patients receiving unfractionated heparin. Unfortunately, when the two are compared directly, there is very poor correlation between the concentration of heparin and the resulting APTT. There is no dose-response relationship between the amount of heparin and the APTT.

The APTT has been used for the monitoring of heparin since the 1950s. The general "rule of thumb" is a therapeutic interval of one-and-a-half to two-and-a-half times the upper limits of the reference range for the APTT. Problems in the monitoring of heparin are based on:

- Variability of heparin
- Patient response
- Reagent
- Instrument¹²⁶

It is necessary for each laboratory to determine the responsiveness of the instrument/reagent method for the APTT to heparin in the patients' specimens. This process needs to be done each time there is a change in the reagent or instrument used in the laboratory.

The concentration of heparin in the blood can be reflected by assays that take advantage of the neutralization of the activity of thrombin or factor Xa.¹²⁷ These assays can use either

a clot endpoint or chromogenic substrate with spectrophotometric endpoint. The assays provide the enzyme (thrombin or activated FX), the inhibitor (AT), and a marker (fibrinogen in the plasma or chromogenic substrate). The rate or amount of conversion of the substrate is inversely proportional to the concentration of heparin present in the specimen.¹²⁷

All approaches to monitoring heparin therapy have their advantages and disadvantages. The dominating methods being used at the present time are the APTT and the chromogenic assay using the inhibition of factor Xa.¹²⁷ The APTT has the advantage of being inexpensive (one-tenth the cost of the Xa inhibitory assay), can be rapidly and easily performed by many different technologists in the laboratory, is available at all hours, and reflects the anticoagulant activity of heparin in the patient's specimen.

The heparin assay has the advantage of being reproducible with more precise therapeutic intervals, making clinical management less problematic. Disadvantages include the cost and a more technically demanding assay technique than the APTT. Some clinicians are also concerned that a target concentration approach may not accurately reflect the state of anticoagulation due to the numerous variables influencing individual patient response.

Low Molecular Weight Heparin

Low molecular weight heparin (LMWH) is prepared from unfractionated heparin by fractionation or depolymerization, which produces heparins of a more uniform molecular mass (ranging from 1 to 12 kD; averaging 5 kD) and function.¹²⁸ They tend to have a greater effect on the inhibition of factor Xa than on other enzymes. A significant advantage of LMWH is that it is administered subcutaneously and does not require therapeutic monitoring in most patients.¹²⁸ Dosing is generally once or twice daily and can be administered by patients, similar to the way diabetic patients administer their own insulin.

It appears that LMWH will replace unfractionated heparin in many clinical circumstances where heparin is required. These products are still not satisfactory for use in extracorporeal circulation (cardiopulmonary bypass or extracorporeal membrane oxygenation).¹²⁸ In addition, despite the fact that many patients do not require monitoring, those of unusually low or high body weight, patients with renal failure, pediatric patients, and patients with other complicating medical conditions that may influence heparin metabolism require careful ongoing evaluation. Monitoring of LMWH requires the target concentration strategy because the therapeutic concentrations do not influence the APTT or other coagulation tests in a dose-dependent way.¹²⁸ These tests may, however, be prolonged during therapy, and this should be noted in testing situations before assigning a diagnosis of a hemostatic disorder.

In monitoring heparin, a natural inhibitor of heparin, called PF4, is released from platelets. The presence of platelets in the specimen or the activation of platelets during collection can thus lead to a false lowering of heparin assayed in the specimen.¹²⁶ Therefore, care should be taken to collect a high-quality specimen, and its preparation should include ensuring that the specimen has a very low platelet count. In addition, if plasma from a heparinized sample sits on cells, the

PF4 can neutralize the heparin, resulting in a falsely decreased APTT.¹²⁶

Vitamin K Antagonists

Vitamin K plays a key role in the post-ribosomal modification of many of the serine proteases. Vitamin K is a cofactor for a carboxylase that inserts an additional carboxyl group on the glutamic acid residues in the amino terminal end of coagulation factors II, VII, IX, and X in addition to PC and PS. This process is a kinetic one in which vitamin K is recycled and can be reused for the carboxylation of multiple molecules through two enzymatic steps.

Coumadin or warfarin acts by interfering with the recycling of vitamin K after it has performed its carboxylation function. This makes warfarin interesting in two respects: (1) Warfarin is not truly an anticoagulant; it leads to the production of coagulation factors that have reduced anticoagulant function (adding warfarin to a tube of blood does not inhibit coagulation). (2) Warfarin is a blood thinning medication and functions to slow down your body's process of forming clots.¹²⁹

Because warfarin functions by altering the synthesis of coagulation factors, the synthetic rate of coagulation factors is variable. In most patients, it takes 5 to 7 days of warfarin therapy before a stable anticoagulant effect can be achieved.¹³⁰

There is a wide degree of variability in the response of thromboplastin reagents and instruments that were used for the PT when evaluating patients receiving warfarin therapy. It has been possible to overcome some of the difficulties with this variability by normalizing the responses of thromboplastin reagents against an international standard.¹²⁶ This process is referred to as the *international normalized ratio (INR)*. The INR is described by the following formula:

$$\text{INR} = \left\{ \frac{\text{PT}_{\text{pat}}}{\text{PT}_n} \right\}^{151}$$

in which the INR is the international normalized ratio, PT_{pat} is the patient's prothrombin time, PT_n is the prothrombin time that reflects the geometric mean of the reference range, and ISI is the international sensitivity index. The ISI needs to be developed for each thromboplastin reagent and instrument combination used in performing prothrombin times and calculation of the INR. Ideal reagents for performing the INR are those that have ISIs below 1.7. Some reagents are exquisitely sensitive, having an ISI of 1 or less, leading to other problems with accuracy and precision in specimens with high INR. The optimal reagent would have an ISI of 1.3 to 1.5.¹²⁶

When monitoring patients who are taking oral anticoagulant therapy, the INR is maintained between 2 and 3.¹²⁶ The exceptions to this therapeutic interval include patients who have a complicating lupus anticoagulant in whom a higher INR may be indicated and patients who have cardiac valves in whom the INR is recommended to be 2.5 to 3.5.¹²⁹

The greatest difficulty in managing oral anticoagulation in patients with thrombosis is the large number of medications and other factors that can increase or decrease the metabolism of warfarin. This includes consumption of foods containing large quantities of vitamin K or antibiotics that kill the normal gut

flora that produce vitamin K enterically. These interactions are important considerations for clinicians who are treating patients.

ADVANCED CONTENT

Warfarin dose-response may be regulated at the transcriptional level. The variants in the gene encoding vitamin K epoxide reductase complex 1 (*VKORC1*) has been described that may explain differences in dose requirements among patients of different ethnicities.^{130,131} Genetic polymorphisms (i.e., CYP2C9) have been demonstrated in patients with resistance to warfarin.¹³¹

Direct Oral Anticoagulants (DOACs)

Most currently used DOACs are factor Xa inhibitors. DOACs include fondaparinux, edoxaban, rivaroxaban, and apixaban.¹³² DOACs are designed to be given in fixed doses without routine monitoring. They have a lower tendency than vitamin K antagonists for food and drug interactions.¹³² The benefits of DOACs are their ease of use and superior safety since they have a lower risk of life-threatening bleeding.¹³²

ADVANCED CONTENT

These DOACs directly inhibit FXa; Rivaroxaban and apixaban are the most commonly employed for stroke prevention in atrial fibrillation and treatment of VTE.¹³² Rivaroxaban is primarily cleared by the kidneys while apixaban, edoxaban, and betrixaban have both renal and hepatic/biliary clearance.¹³³ (Please note that betrixaban was discontinued in 2020.)

Fondaparinux sodium (Arixtra®) is the first synthetic pentasaccharide and an inhibitor of factor Xa.¹³⁴ It is approved for the prophylaxis and treatment of deep vein thrombosis (DVT) and pulmonary embolism (PE). In terms of prevention, it is widely prescribed for venous thromboembolic events (VTE) in postoperative adults undergoing major orthopedic and abdominal surgery.¹³⁴ Fondaparinux sodium is also approved for use in the following conditions: individuals at a high risk for VTE who are immobilized due to acute illness, acute symptomatic superficial vein thrombosis of the lower limbs, and acute coronary syndromes.¹³⁴ While heparins and heparinoids are prepared from animal extracts, fondaparinux is synthesized chemically, ensuring no general supply issues and minimal risk of contamination. "Fondaparinux has 100% absolute bioavailability subcutaneously, instant onset of action, a long half-life of 17–21 hours, direct renal excretion, and fewer adverse reactions when compared with other direct oral anticoagulants."¹³⁴ For direct Xa inhibitors, anti-Xa assays with a drug-specific calibrator are the most accurate and more widely available options (aside from quantitative mass spectrometry).¹³⁴ Since direct Xa inhibitors will show positive results for anti-Xa assays that are not calibrated to the specific

medication, screening with an anti-Xa heparin calibrated assay may be able to give qualitative assessment of presence or absence.¹³⁵ PT/INR may also be used if these are unavailable but is extremely variable in its sensitivity to the medications depending on reagent sets.^{135,136}

Caution should be taken when evaluating samples for coagulation testing on patients who are currently on anticoagulation. Although many reagent sets include binding or enzymatic reversal of heparins, most commercially available reagents do not include reversal for the effects of DOACs.^{137,138} This may lead to false-negative or false-positive results. Adexanet alfa is FDA-approved as a reversal agent for rivaroxaban and apixaban.¹³⁷ It is an inactive FXa, which is unable to cleave prothrombin. However, prothrombin complex concentrates, recombinant FVIIa, and hemodialysis may also be considered for patients in whom reversal is indicated.¹³⁷

Others in development include the use of tissue factor, factor VII, factor V, and factor VIII as potential targets.

However, measurement may be necessary in certain situations (e.g., compliance with medication, emergency situations, invasive procedures, bleeding, thrombosis, changes in renal/hepatic function, extremes of age and/or weight). The gold standard method for DOAC measurement is liquid chromatography-mass spectrometry (LC-MS/MS), but it is time consuming and requires expensive equipment.¹³³ "The general consensus for the assessment of a DOAC is clotting or chromogenic assays using specific standard calibrators and controls."¹³³

Direct Thrombin Inhibitors (DTIs)

Dabigatran is a DOAC that is a direct thrombin inhibitor and is administered orally.¹³² Because some patients cannot tolerate heparin, especially those with HIT, additional anticoagulants are currently being used in a limited way. Direct thrombin inhibitors (DTIs) inhibit thrombin through the binding of the active-binding site of thrombin. They prevent blood clots from forming in the body. Dabigatran and its metabolites are competitive, direct thrombin inhibitors, working on both free and fibrin-bound thrombin by interaction with the thrombin-active site.¹³⁹ This anticoagulation, taken orally, reaches peak levels 1 or 2 hours after administration.¹³⁹ Elimination of the medication is primarily renal, leading to the need to monitor renal function upon initiation of therapy, as well as during therapy in patients at risk for developing decreased renal function (e.g., SLE or renal toxic medications).¹³⁹ Eighty percent of unchanged dabigatran is eliminated by renal clearance.¹³⁹

Currently, recommendations for assessment of dabigatran anticoagulant effect include dilute thrombin time or ecarin-based (clot-based or chromogenic) assays.¹³⁹ TT may be used if these are unavailable, especially given that the TT is extremely sensitive to the presence of the drug. Dabigatran has an FDA-approved reversal agent, idarucizuma.¹⁴⁰ However, prothrombin complex concentrates, recombinant FVIIa, and hemodialysis may also be considered for patients in whom reversal is indicated.¹³⁹

ADVANCED CONTENT

Direct thrombin inhibitors administered intravenously include hirudins, bivalirudin, argatroban, and G-quadruplex DNA aptamers.¹³⁸ Natural Hirudin, an anticoagulant produced in the salivary glands of leeches, is a direct thrombin inhibitor that binds irreversibly in a 1:1 stoichiometric relationship with high affinity to thrombin.¹⁴¹ It has a plasma half-life of approximately 1 hour. It has no structural similarity to heparin and does not cross-react with heparin-dependent antibodies. Recombinant hirudin (rH) is available for treatment of HIT.¹⁴¹ A cell-free protein synthesis approach, which can express rH with higher antithrombin activity was reported in 2020. Previous rHs were produced by bacterial or yeast expression systems.¹⁴¹ "Although these methods can increase the expression of recombinant hirudin, the thrombin inhibition constant is still lower than that of natural hirudin."¹⁴¹

Hirudin therapy is commonly monitored using the APTT, with a target value of 1.5 to 2.0 times the median of the normal range.¹⁴¹ However, variable responses of APTT reagents to hirudin can be problematic. Daily monitoring is recommended because hirudin is predominantly cleared by the kidneys. Therefore, monitoring is especially important in patients with impaired renal function.¹⁴¹

Argatroban is a synthetic thrombin L-arginine derivative inhibitor that functions similarly to hirudin but is cleared even in patients with moderate to severe renal failure.¹⁴² It shows reversible inhibition and has a half-life of approximately 45 minutes. It is a drug of choice in HIT patients, especially in patients in whom hirudin cannot be used (renal failure or risk of anaphylaxis) with a target value of 1.5 to 3.0 times the median of the normal range.¹⁴² The drug should be used cautiously in patients with liver disease.

Bivalirudin is a reversible synthetic thrombin inhibitor that can be used as an alternative drug in patients with HIT. It contains a C-terminus similar to hirudin that binds thrombin's exosite 1 and an amino terminus that binds thrombin's active site.¹⁴³ It is administered intravenously and has a half-life of 25 minutes.¹⁴³ The current use is in coronary angioplasty for patients who cannot use heparins.¹⁴⁴ DTIs can be monitored with anti-IIa chromogenic assays.^{134,135}

Antiplatelet Agents

Used alone or in combination with other anticoagulants, a number of antiplatelet function agents have been developed. By far, the most commonly used agent is aspirin. It functions by inhibiting prostaglandin synthesis. Its action has been well recognized for over 20 years, and its use (in low doses) for prophylaxis to prevent stroke and MI has been well characterized. Aspirin has been used in combination with heparin or LMWH in some patients who have arterial thrombotic events.¹⁴⁵

Antiplatelet agents are used for patients with ischemic heart disease, cerebrovascular disease, and peripheral artery disease

(PAD) since they have been proven to significantly reduce the mortality and morbidity.¹⁴⁵ "In patients with acute coronary syndromes (ACS) and in those undergoing angioplasty with stent implantation dual antiplatelet therapy with aspirin and an adenosine diphosphate (ADP) receptor antagonist is indicated."¹⁴⁶ Other antiplatelet agents are likely to be developed in the near future. Their effectiveness is gradually increasing, as clinicians become more accustomed to their application. In general, these antiplatelet agents have not required laboratory monitoring.

Thrombolytic Therapy

Vascular surgeons and cardiologists are becoming increasingly adept at using thrombolytic agents to lyse clots formed in coronary arteries and other vessels. The site of these thrombi can also be stented with devices that hold the lumen of the artery open and allow recirculation beyond the previous occlusion. The difficulty occurring with these therapies has been prevention of recurrence of thrombosis of the vessel.¹⁴⁷ The most promising agents used in a post-thrombolytic setting or with stents have been inhibitors of platelet function with or without heparin. Thrombolytic therapies using urokinase and streptokinase have been available for more than two decades and tissue plasminogen activator is another fibrinolytic therapy used for acute pulmonary embolism.¹⁴⁸⁻¹⁵⁰ In any event, streptokinase is still a significant agent in treatment of cardiovascular diseases.¹⁵¹

The medications that convert plasminogen to plasmin without fibrin as a cofactor may be classified in two

categories: (1) agents that convert plasminogen to plasmin directly and (2) agents that are fibrin-specific. In the former group are streptokinase or urokinase. The latter group includes tissue plasminogen activator (tPA).¹⁵⁰

The only FDA approved treatment for patients with acute ischemic stroke is thrombolysis with intravenous tissue plasminogen activator (tPA), but its use is limited by a narrow therapeutic window, selective efficacy, and hemorrhagic complication.^{150,151}

Because the goal is the lysis of fibrin clots, agents that are specific for fibrin would logically be preferred. Streptokinase and urokinase cause a hypofibrinogenemia secondary to fibrinogenolysis in virtually all patients, even when the medication is given by a catheter into the specific artery containing the clot. The advantage of the fibrin-specific agents is that it is feasible to administer these agents systemically into the vessel containing the thrombus in a catheter-directed fashion. In fact, tPA can bind to circulating fibrin degradation products, activate plasmin, and cleave fibrinogen. Therefore, although less frequent and less severe, fibrin-specific agents can be complicated by hypofibrinogenemia. In a comparison of low-dose versus standard-dose intravenous recombinant tPA in patients with acute ischemic stroke, equivalent results were reported in terms of effectiveness and safety.¹⁵² However, the study showed that a lower dose had a lower risk of intracerebral hemorrhage.^{152,153}

More recently, other thrombolytic agents have become available. Enzymes including alteplase, reteplase, tenecteplase, urokinase, streptokinase, and anistreplase are FDA approved for the treatment of cardiovascular diseases.^{154,155}

SUMMARY CHART

- Endothelial cells function to facilitate platelet adhesion and platelet activation at the site of injury, secrete von Willebrand's factor, and secrete an inhibitor of tissue plasminogen, which suppresses fibrinolysis.
- Natural anticoagulants in plasma include antithrombin (AT), heparin cofactor II (HC-II), protein C (PC), protein S (PS), and tissue factor pathway inhibitor (TFPI).
- Antithrombin is a major inhibitor of thrombin and factor Xa.
- Protein C is a vitamin K-dependent zymogen that, once activated by thrombin, proteolytically degrades factors VIIIa and Va, two of the major cofactors involved in thrombin generation.
- Protein S is a vitamin K-dependent zymogen that is a major cofactor for protein C.
- Inhibitors of plasmin include α_2 -antiplasmin, α_1 -antitrypsin, α_2 -macroglobulin, AT, and C1 esterase inhibitor.
- Inhibitors of plasminogen include plasminogen activator inhibitor-1 (PAI-1), PAI-2, and PAI-3, with PAI-1 being the most significant inhibitor of tissue plasminogen activator (tPA).
- Inherited thrombophilia is a group of congenital hematology disorders that includes a variety of hypercoagulable states that usually present as venous or arterial thrombosis, or both.
- Activated protein C resistance (APC-R) can be defined as deficient anticoagulant response of plasma to the addition of APC. Causes of APC-R include inhibitors to APC, functional PS deficiency, and mutated forms of factor V and VIII molecules; factor V Leiden is the most common cause of APC-R.
- The prothrombin nucleotide G20210A mutation can be described as a single guanine to adenine mutation at nucleotide position 20210 and is associated with increased risk of deep vein thrombosis and elevated prothrombin levels.
- Antiphospholipid (aPL) syndrome is an acquired thrombotic disorder. Laboratory evidence of aPL antibodies can be in the form of either immunologically demonstrated aPL antibodies; enzyme-linked immunosorbent assay (ELISA) for anticardiolipin and beta-2 glycoprotein I antibodies, and/or lupus anticoagulant.

SUMMARY CHART—cont'd

- The APTT may be prolonged in heparin therapy or contamination, insufficient volume of sample, disseminated intravascular coagulation (DIC), factor deficiencies, and antibodies directed against factors in intrinsic or common pathways.
- Immune-mediated heparin-induced thrombocytopenia is described as a sudden decrease in the platelet count 5 days after heparin therapy is initiated and is caused by the interaction of platelet factor 4 (PF4) on the platelet membrane.
- Unfractionated heparin acts by accelerating the rate at which AT is capable of binding thrombin. Heparin therapy may be monitored by the APTT, with a target value of one-and-a-half times the upper limits of the reference range for the APTT. Alternatively, a heparin-calibrated anti-Xa chromogenic assay may be employed.
- Low molecular weight heparin (LMWH) is prepared from unfractionated heparin by fractionation or depolymerization, producing heparins of 1,000 to 12,000 daltons. They inhibit factor Xa more efficiently and do not require therapeutic monitoring.
- Warfarin therapy acts by interfering with the recycling of vitamin K and depressing the activities of factors II, VII, IX, and X; PC; and PS. Vitamin K is a cofactor for a carboxylase that inserts an additional carboxyl group on the glutamic acid residues in the amino terminal end of coagulation factors II, VII, IX, and X.
- Warfarin therapy is monitored by the prothrombin time (PT) and international normalized ratio (INR); the INR is maintained between 2 and 3 for patients on anticoagulant therapy.
- Direct oral anticoagulants (DOACs) reversibly inhibit either Thrombin (dabigaTran) or FXa (rivaroxaban, apixaban, edoxaban, betrixaban). These anticoagulants are not routinely monitored.
- Thrombolytic agents include tPA, urokinase, and streptokinase. Hemostatic evaluation includes PT, APTT, fibrinogen assay, thrombin time, and platelet count.

CASE STUDY 29-1

A 40-year-old man presented to the emergency department with a swollen, painful right leg of 3 days' duration and shortness of breath for the past 12 hours. He also had sharp pain in the left side of his chest when he took a deep breath.

PERTINENT HISTORY

Past Illnesses: Had pneumonia 8 years ago with full recovery.

Occupation: Cross-country truck driver.

Social Habits: Drinks alcohol occasionally on a social basis. Has smoked a pack of cigarettes a day for 22 years.

Family History: Mother died of "clots in her lungs." Father and siblings are alive and well. One sister had a "clot in her leg" after she delivered her only child.

PERTINENT PHYSICAL FINDINGS

Vital Signs: Blood pressure, 110/75 mm Hg; respirations, 20/min; pulse, 72/min and regular; temperature, 38°C; weight, 130 kg.

Chest: Distant breath sounds on the left with dullness to percussion. Heart examination is not remarkable.

Bones, Joints, and Muscle: Right leg, ankle, and foot are slightly edematous and tender to palpation.

LABORATORY FINDINGS

Hgb, 14.5 g/dL; WBC, 12,000/ μ L (\uparrow); differential WBC: Neutrophils, 9100/ μ L (\uparrow); lymphocytes, 2200/ μ L; monocytes 500/ μ L; eosinophils, 200/ μ L; platelet count; 220,000/ μ L

PT, 12 seconds; INR, 1.0; APTT, 22 seconds; fibrinogen, 269 mg/dL; thrombin time, 18 seconds; D-dimer, 500 ng/mL (\uparrow)

Cholesterol, 230 mg/dL (\uparrow), HDL, 25 mg/dL (\downarrow)

QUESTIONS

1. What risk factors for thrombophilia are present in this patient?
2. The elevated D-dimer could indicate which condition(s)?
3. What is the differential diagnosis?
4. What laboratory tests could be helpful in determining the etiology of the condition (a) in the acute setting? (b) after cessation of anticoagulation?
5. What therapy is indicated?
6. How is the therapy monitored?

ANSWERS

1. Risk factors for thrombophilia in this patient include past lung infection, sedentary job, alcohol consumption, smoking, and family history of thrombosis.
2. The elevated D-dimer is indicative of thrombosis.
3. The patient history, clinical presentation, and lab findings are suggestive of venous thromboembolism (VTE) or DVT with pulmonary embolism (PE).
4. Laboratory tests that could be helpful in determining the etiology of the condition in the acute setting include activated protein C resistance, factor V Leiden (FVL)

Continued

CASE STUDY 29-1—cont'd

by PCR, prothrombin gene mutation 20210 by PCR, MTHFR by PCR, and possibly anticardiolipin. Laboratory tests that could be helpful in determining the etiology of the condition after recovery and conclusion of anticoagulation include protein C, protein S, and AT. All of these were performed, and the patient was found to be heterozygous for FVL only.

5. Heparin therapy (unfractionated heparin or LMWH) is indicated immediately to treat DVT/PE and then oral anticoagulant for long-term therapies.
6. Therapy is monitored via APTT and PT/INR.

CASE STUDY 29-2

A 28-year-old woman presents with pain in the left leg. A screening test in the ED confirms that she has a large thrombosis. She is married with no children.

Current Medications: Daily oral contraceptives.

Family History: Parents and siblings (all male) are alive and well with no previous thrombotic symptoms.

PERTINENT HISTORY

Hematopoietic: No history of anemia, bleeding disorder, jaundice, or easy bruising.

Menstrual History: Unremarkable menstrual cycle, although somewhat irregular.

PERTINENT PHYSICAL FINDINGS

Vital Signs: Blood pressure, 125/80 mm Hg; respirations, 16/min; pulse, 72/min, and regular.

LABORATORY FINDINGS

Hgb, 13.5 g/dL; WBC, 8,000/ μ L; differential WBC:

Neutrophils, 5100/ μ L; lymphocytes, 2200/ μ L; monocytes, 500/ μ L; eosinophils, 200/ μ L; platelet count, 100,000/ μ L.

PT, 12 seconds; INR, 1.0; APTT, 70 seconds (\uparrow); fibrinogen, 269 mg/dL; thrombin time, 18 seconds.

QUESTIONS

1. What is this patient's risk factors for thrombosis?
2. The elevated APTT could be a clue to which inherited thrombophilia?
3. Why would this patient exhibit thrombotic symptoms but their siblings never have?

ANSWERS

1. The patient's use of oral contraceptives is a risk factor for thrombosis.
2. The elevated APTT could be a clue to APC-R.
3. In APC-R, females are often only symptomatic when there are increased levels of estrogen, such as with oral contraceptive use and pregnancy.

REVIEW QUESTIONS

1. Which of the following factors would put a patient at risk for thrombosis?
 - a. A factor deficiency
 - b. Regular movement/exercise
 - c. Hypertension
 - d. Minimal use of drugs
2. The risk of activation of coagulation by the endothelium is higher when which of the following conditions is present?
 - a. When the vessel lumen is wide, allowing blood to pass easily and at slower velocity
 - b. When the vessel lumen is narrow and blood is passing at high velocities
 - c. When the vessel lining is intact and there is no endothelial cell exposure
 - d. When platelets are circulating inactively within the blood
3. Thrombin is responsible for which of the following activities?
 - a. Stabilizing the platelet plug into a fibrin clot
 - b. Enabling the bond of vWF during primary hemostasis
 - c. Activating platelets
 - d. Breaking down fibrin strands to begin fibrinolysis
4. Antithrombin is a cofactor with which of the following elements?
 - a. Collagen
 - b. Heparin
 - c. Thrombin
 - d. Tissue factor
5. Antithrombin inhibits which of the following factors?
 - a. FVIIIa
 - b. FVIIa
 - c. FXa
 - d. FXIIIa

REVIEW QUESTIONS—cont'd

6. Protein C inhibits which of the following factors?
 - a. FVIIIa
 - b. FVIIa
 - c. FXa
 - d. FXIIIa
7. Protein C requires the presence of a cofactor, which is
 - a. Antithrombin
 - b. Protein S
 - c. Calcium
 - d. Thrombin
8. Protein C and protein S require the presence of which of the following to function?
 - a. Calcium
 - b. Vitamin E
 - c. Potassium
 - d. Vitamin K
9. A young female patient taking oral contraceptives and experiencing thrombosis is more likely to be diagnosed with which of the following?
 - a. Protein C deficiency
 - b. AT deficiency
 - c. APC-R
 - d. Protein S deficiency
10. Neonatal purpura fulminans is more likely to be seen in which of the following inherited conditions?
 - a. APC-R
 - b. PT mutation
 - c. Protein C deficiency
 - d. AT deficiency
11. aPL syndrome requires which of the following findings?
 - a. dRVVT and a second phospholipid-dependent clotting assay
 - b. Only one uncorrected mixing study
 - c. Abnormal PT or aPTT only
 - d. Only an abnormal factor analysis
12. aPL syndrome has a documented history of prevalence with which other condition?
 - a. APC-R
 - b. SLE
 - c. AT deficiency
 - d. PT mutation
13. HIT develops in 1% to 5% of hospital patients exposed to heparin for how long?
 - a. 1 to 2 days
 - b. 5 days
 - c. 1 to 2 weeks
 - d. More than 3 weeks
14. Which lab results are consistent with HIT?
 - a. Platelet count increases
 - b. Platelet count decreases
 - c. Prolonged PT
 - d. Prolonged aPTT
15. In HIT, IgG antibodies are formed to which of the following?
 - a. vWF-platelet complex
 - b. aPL antigens
 - c. Heparin-PF4 complex
 - d. FVIII-PF4 complex
16. Which of the following patients is at highest risk of thrombosis?
 - a. A woman of reproductive age with heterozygous FVL not taking oral contraceptives
 - b. A diabetic patient with healthy kidney function
 - c. An ALL patient taking L-asparaginase for chemotherapy treatment
 - d. A hospitalized patient receiving heparin postsurgery for 2 days only
17. Testing for genetic abnormalities would contribute to which of the following reasons for lab analysis for hypercoagulable states?
 - a. Identifying risk factors for thrombosis
 - b. Modifying treatment regimens
 - c. Preventing thromboembolic disease
 - d. Discovering an underlying disease that can be treated
18. The D-dimer assay is utilized to determine whether which of the following conditions is present?
 - a. APC-R
 - b. Factor deficiency
 - c. VTE
 - d. aPL syndrome
19. Which of the following vitamin-K antagonist used to treat hypercoagulable states?
 - a. LMWH
 - b. Hirudin
 - c. Argatroban
 - d. Warfarin
20. Which lab assay is used to monitor heparin therapy?
 - a. PT
 - b. aPTT
 - c. FVIIa assay
 - d. Protein C

See answers at the back of this book.

SELECT LABORATORY METHODS

CHAPTER 30

Body Fluid Examination

Analysis of Serous, Cerebrospinal, and Synovial Fluids

Sharon L. Schwartz, MS, MLS(ASCP)SH

CHAPTER OUTLINE

Types of Body Fluids and Anatomy

Serous Fluids: Pericardial, Pleural, and Peritoneal
Cerebrospinal Fluid
Synovial Fluid

Specimen Collection, Preparation, and Analysis

Collection
Preparation
Laboratory Analysis and Clinical Correlations

Cellular Components of Body Fluids

Neutrophils
Lymphocytes

Macrophages

Tissue Cells
Eosinophils, Basophils, and Mast Cells

Serous Fluids: Pleural, Pericardial, and Peritoneal

Effusions: Transudates and Exudates
Cellular Responses, Microorganisms, and Malignant Cells

Types of Effusions, Laboratory Analysis, and Clinical Correlations

Pleural and Pericardial Effusions
Peritoneal Effusions

Cerebrospinal Fluid

Specimen Collection and Processing

Laboratory Analysis and Clinical Correlations

Synovial Fluid

Specimen Collection and Processing
Laboratory Analysis and Clinical Correlations
Crystal Analysis and Clinical Correlations
Artifacts

Summary Chart

Case Study 30-1

Case Study 30-2

Case Study 30-3

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 30-1 List the types of body fluids from closed body cavities that are studied in the hematology laboratory.
- 30-2 Identify the type of procedure used to obtain each type of body fluid.
- 30-3 Evaluate the formation of serous (peritoneal, pericardial, and pleural), cerebrospinal, and synovial fluids.
- 30-4 Assess the cell types normally found in serous, cerebrospinal fluid, and synovial fluids.
- 30-5 Analyze effusion, transudate, and exudate and their characteristics.
- 30-6 Assess the laboratory methods for body fluid analysis.

- 30-7 Evaluate the principle, advantages, and disadvantages of the cytocentrifuge method for body fluid morphological examination.
- 30-8 Describe the common cellular artifacts introduced by cytocentrifugation.
- 30-9 Distinguish the difference between a traumatic lumbar puncture from a true cerebral spinal hemorrhage.
- 30-10 Assess the purpose of synovial fluid analysis.
- 30-11 Associate the types of crystals found in synovial fluid with the related joint diseases.
- 30-12 Define birefringence as it relates to crystal analysis.

The analysis of body fluid from normally sterile body compartments is an important element of patient care that provides clinicians with valuable information in the diagnosis and treatment of disease. Under the abnormal conditions of disease, these fluids can significantly increase in volume and contain infectious and/or inflammatory substances and cellular elements. This chapter describes the hematological analysis of the most commonly encountered body fluids and discusses the qualitative, quantitative, morphological, and cytological analyses, when appropriate, that are performed by respective laboratories.

The hematology laboratory is responsible for cell enumeration (or cell count) and morphological evaluation (or differential) of body fluid specimens. The types of cells and their concentrations are useful in disease diagnosis. The cells that can be found include white blood cells (WBCs), red blood cells (RBCs), tissue cells, and tumor cells. It is important for laboratory professionals to be able to distinguish benign from reactive or malignant cells and appropriately refer these findings to a pathologist. Crystal formation in body fluids is also diagnostically significant.

Types of Body Fluids and Anatomy

Body fluids, an all-inclusive term, may be used to describe a diverse group of nonblood, nonurine fluids that are found in closed body cavities: the thoracic and abdominal cavities, central nervous system, and joint spaces. Fluids from the thoracic and abdominal cavities are referred to as **serous fluid**; that is, they are related to or resemble serum in their composition. In reality, they are ultrafiltrates of plasma.

Serous Fluids: Pericardial, Pleural, and Peritoneal

The thoracic (chest) cavity contains the heart and lungs. The heart is enclosed within a fibrous structure, known as the **pericardial sac**. The pericardial sac is lined by a single layer of mesodermal cells called **mesothelial cells**. These cells form a continuous membrane, known as the **pericardium**, that covers the parietal (inner) surface of the pericardial sac and the visceral (outer) surface of the heart. The potential space between the two membranous surfaces is called the **pericardial cavity**. It contains a small amount of serous fluid that is secreted by the parietal pericardium and absorbed by the visceral pericardium, functioning as a lubricant between the membranes as the heart relaxes and contracts.

The lungs are encased by a thin membrane composed of a single layer of mesothelial cells known as the **pleura**. The lung surface is covered by the visceral pleura. The pleura also lines the inside surface of the chest wall, forming the parietal pleura. The potential space between these surfaces forms the **pleural cavity** that contains a thin film of serous fluid produced by the parietal pleura and absorbed by the visceral pleura. This fluid provides lubrication for the membranes during respiration while preventing the formation of any space between the lungs and chest wall. This maintains the lungs in an expanded state.

The abdominal cavity is lined by a membrane also composed of a single layer of mesothelial cells known as the **peritoneum**. It is the largest and most complex serous membrane

in the body. The parietal peritoneum covers the inner surface of the abdominal wall. The visceral peritoneum covers the surfaces of the stomach, small and large intestines, liver, and the superior aspects of the urinary bladder and uterus. The kidneys, pancreas, duodenum, some lymph nodes, and the abdominal aorta are posterior to (not enclosed by) the peritoneum and are known as **retroperitoneal**. The potential space created by the two layers of peritoneal membranes is known as the **peritoneal cavity**. The parietal peritoneum secretes a serous fluid that prevents friction between the membranes from the constant motion of the abdominal organs and is absorbed by the visceral peritoneum. Normal pleural, pericardial, and peritoneal cavities do not contain appreciable amounts of fluid and, as such, are not true cavities until a disease state causes the accumulation of fluid.

Cerebrospinal Fluid

The central nervous system (CNS) consists of the brain and spinal cord, enclosed by the bony structures of the skull and vertebrae of the spine. These structures are lined by special membranes known as the **meningeal membranes** or **meninges**. The meninges consist of three layers of tissue: a relatively thick outer membrane known as the **dura mater**, which provides the major protection for the brain and spinal cord; a thinner middle membrane known as the **arachnoid mater**; and an inner membrane that lies directly on the surface of the brain and spinal cord known as the **pia mater**. The area between the arachnoid mater and pia mater is known as the **subarachnoid space**.¹ It contains a selective ultrafiltrate of plasma known as **cerebrospinal fluid (CSF)**, which protects and supports the brain and spinal cord and maintains a constant ionic environment by circulating nutrients and removal of waste products. These functions are controlled by the blood-brain barrier, which consists of the endothelial cells of brain capillaries wrapped by supporting cells of the brain known as **astrocytes**. These cells permit the passage of essential substances such as water, oxygen, and carbon dioxide, and exclude the passage of large molecules such as proteins, peptides, and many drugs.

The CSF circulates through the ventricular system of the cerebrum, cerebellum, and brainstem. Masses of specialized capillaries in the pia mater, known as the **choroid plexus**, project into the ventricles and are the main source of CSF production. The ventricles and central spinal canal are lined by a layer of epithelial cells with villous projections and cilia known as **ependymal cells**, or the **ependyma**. Together, the ependyma and choroid plexus produce approximately 20 mL of CSF per hour, that is reabsorbed at the same rate by the **arachnoid villi** of the arachnoid mater. The arachnoid mater penetrates the inner dura and its venous sinuses. The choroid plexus epithelium and the endothelium of the capillaries in contact with the CSF form the blood-CSF barrier.¹ This barrier controls the concentrations of solutes between the blood and CSF. The normal total volume of CSF in adults is approximately 90 to 150 mL and approximately 10 to 60 mL in neonates.

Synovial Fluid

Joints, also known as **articulations**, are the junctions between two or more bones. The freely movable limb joints of the body (e.g., shoulder, elbow, knee, hip, etc.) are enclosed by **synovial**

fibrous tissue for support and alignment of the bones, known as the **joint capsule**. It consists of an outer fibrous layer and an inner membrane with a rich blood supply, known as the **synovial membrane** or **synovium**, composed of a single layer of mononuclear **synovial cells**. Articular cartilage covers the bony surfaces of the joint, permitting smooth motion during movement. The synovium also surrounds the tendons and free margins of the ligaments and articular cartilage, but it does not extend over the surface of the intra-articular cartilage. An ultrafiltrate of plasma, known as **synovial fluid** (or **mucin**; an older terminology), is secreted by the synovial cells and transports nutrients to the articular cartilage. This fluid fills the **synovial cavity**, the space that exists between the bones and is enclosed by the synovium and intra-articular cartilage. The synovial cells also produce a mucopolysaccharide known as **hyaluronic acid**. This gives the fluid a viscous consistency, providing lubrication and facilitation of movement. Synovial cells are also phagocytic, removing cellular debris that exists at the surface of the membrane. The large joints may normally contain approximately 1 mL of synovial fluid.

Specimen Collection and Preparation

A properly collected and prepared specimen is crucial to accurate diagnosis and treatment. Body fluid extraction procedures are invasive and carry the potential to cause harm to patients. It is critically important to follow laboratory protocol during each step of the process.

Collection

Body fluids are collected only by a physician or properly trained medical personnel. Specimens are always obtained by aseptic (sterile) technique for diagnostic or therapeutic purposes. They must also be handled with great care, as they frequently cannot be recollected (irretrievable), especially in neonates. Specimens are potentially infectious and must be handled with universal precautions. The aspirated fluid is placed into evacuated, anticoagulated, and/or sterile tubes to be sent to the laboratory. Details about the collection of each body fluid type are discussed in more detail later in the chapter.

Preparation

On receipt in the laboratory, the body fluid specimen must be processed immediately. The cells within a fluid are usually at lower concentrations than peripheral blood and are more fragile; their viability rapidly deteriorates once they are removed from the body, and they begin degenerating, affecting their morphology.

Laboratory Analysis and Clinical Correlations

Qualitative Analysis

First, the specimen is evaluated for the appropriate collection container and visually evaluated for physical properties, including volume, color, clarity, viscosity, and/or the presence of fibrin clot. The quality of the specimen has a direct influence on the quality of laboratory results. Details are addressed under specific fluid analysis in this chapter.

Quantitative Analysis

Next, the sample is prepared for cellular quantification. Historically, most automated methods in use for peripheral blood

cell counts cannot be applied to body fluids for several reasons, including:

1. The peripheral blood cell-counting instruments are not standardized for a body fluid medium.
2. Cell size variation in fluids is greater than in peripheral blood.
3. Background debris and clots are often present in serous fluids.
4. Certain fluids (e.g., synovial) are quite viscous.

However, newer more advanced automated peripheral blood analyzers often have a body fluid (BF) mode. This adds a quantitative, automated procedure for analyzing serous and synovial fluids, providing total nucleated cell (TNC), white blood cell (WBC), and red blood cell (RBC) counts. Selecting the BF mode on the analyzer changes the dilution ratios and reporting format most applicable to the characteristics of these types of specimens. The mode applies the principle of hydrodynamic focusing to improve the accuracy and precision of the cell counts. A semiconductor laser beam is emitted to the cells as they pass through a flow cell in a single file. The size and interior components of cells scatter the light in different directions. The captured light is captured by photodetectors and converted into electrical pulses that are used to determine the cells' size, concentrations, internal complexity, and classifications.

The only exception to automated BF analysis applies to CSF, due to low cell counts that can potentially be less than the particle count (background count) of the analyzer diluent.² It is suggested that these specimens not be processed by automated systems.

Despite its imprecision, manual cell counting using a hemacytometer is the preferred method for cell counts on body fluid specimens (see Chapter 31). Unlike peripheral blood, and unless the specimen is obviously bloody or turbid, the fluid is counted undiluted. If it is necessary to dilute the sample, the diluent used must be appropriate for the type of specimen (i.e., synovial fluid diluent must not contain acetic acid; see discussion on Synovial Fluid.) The diluent must be filtered and free of any background debris by microscopic verification. Separate dilutions may be required for the RBCs and TNCs to reduce error. The number of squares (area) to be counted is determined by the concentration of cells present:³

- If more than 200 cells are seen in a square millimeter of the chamber (e.g., a WBC square), the cells in the five RBC squares of the center square should be counted
- If more than 200 cells are seen in the entire ruled area (i.e., all nine squares; 9 mm²), the cells in each of the four corner WBC squares should be counted.
- If fewer than 200 cells are seen in the entire ruled area, the cells in all nine squares should be counted.

The calculation of the cell count is based on the formula:

$$\text{Total cells}/\mu\text{L} = \frac{\text{number of cells counted} \times \text{dilution factor}}{\text{number of squares counted} \times \text{volume/square}}$$

$$\text{volume/RBC square: } 0.004 \mu\text{L}$$

$$\text{volume/WBC square: } 0.1 \mu\text{L}$$

The same area on both sides of the hemacytometer is counted, and the percent difference of the count from each side must be less than or equal to 10%; if greater, the cellular distribution was poor and the counts must be repeated.

Microscopic and Morphological Analysis

Finally, the sample must be prepared for morphological evaluation. This portion of the body fluid analysis is an extremely important aspect of the process because it becomes the permanent record of the cellular constituents within the specimen and provides the basis for diagnosis. Body fluid slides are prepared by a technique known as **cytocentrifugation** by using a cytocentrifuge: a device that concentrates and deposits the cells within a fluid suspension directly onto a glass slide as a monolayer. The method utilizes a small funnel apparatus that is held against a glass slide by a clamp that fits into the cytocentrifuge. Between the funnel and the slide is a fitted strip of filter paper with a hole under the funnel outlet. Cytocentrifugation is based on the principle that cells are concentrated by slow centrifugation with simultaneous absorption of the noncellular portion of the fluid into a filter paper. The filter preferentially absorbs the fluid portion, leaving the cells in a concentrated button on a microscope slide. This method markedly improves the quality of the cell morphology obtained compared with the direct smear technique.

The College of American Pathologists (CAP) and the Clinical and Laboratory Standards Institute (CLSI) recommends cytocentrifugation as the preferred method for hematologic analysis.³ The advantages of this method are outlined in Box 30-1.

There are, however, certain disadvantages of the technique. Cytocentrifugation can create artifacts which laboratory professionals should be aware of (Box 30-2). Cytoplasmic artifacts can include irregular fragmentation, projections, localization of granules, and peripheral vacuolization (Figs. 30-1, 30-2, and 30-3).

Even with the disadvantages, the cytocentrifuge method produces a high-quality representation of the cellular elements present in the fluid. The best results are obtained with fresh specimens, processed immediately or as soon as possible after collection. A small amount of added protein, such as a drop of bovine or human serum albumin, will protect cells during cytocentrifugation. The cell count helps to determine the volume of body fluid specimen necessary in performing cytocentrifuge preparation; high cell counts require less sample or predilution. Slides must be prepared regardless of whether any cells were

BOX 30-2 Artifacts From the Cytocentrifugation Method of Body Fluid Analysis

- There can be an overconcentration of cells in fluids with high cell counts.
- Cells in the interior of the cell button appear smaller with denser nucleus than cells at the periphery.
- Abnormal cells are more likely to be affected because of their fragility.
- Nuclear-induced changes can include peripheralization, distorted shape, segmentation, fragmentation, holes, and more obvious nucleoli.
- Cytoplasmic artifacts can include irregular fragmentation and string-like processes, clustering of granules, peripheral vacuolization, and perinuclear zone in lymphocytes and monocytes.

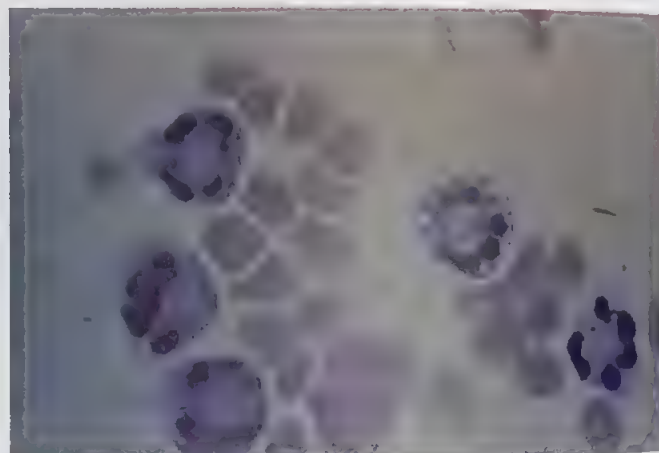


FIGURE 30-1 Artifact of cytocentrifugation. Peripheralization, vacuolization, and hyperlobulation of neutrophils. Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)



FIGURE 30-2 Artifact of cytocentrifugation. Prominence of nucleoli, vacuolization in lymphocytes. Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

BOX 30-1 Advantages of the Cytocentrifugation Method of Body Fluid Analysis

- Ease of preparation
- Speed of preparation
- Concentrates specimen for good cell recovery
- Cellular differentiation is determined by Wright or Wright-Giemsa stain
- Normal, reactive, and malignant cells can be identified
- Cytocentrifuge slide becomes retrievable permanent record



FIGURE 30-3 Artifact of cytocentrifugation. Cytoplasmic projections of lymphocytes. Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

seen in the hemacytometer, as the potential to detect abnormal or malignant cells increases with the cytocentrifugation process. The optimal speed and duration are determined by the individual laboratory based on its equipment design.

After cytocentrifugation, slides are allowed to air-dry and are stained with the Wright (Wright-Giemsa) stain. Slides can also be made for other cytological analyses and staining. The slides are then used to perform the total nucleated cell differential and the detection of any reactive or malignant cells.

Laboratories without cytocentrifugation equipment need to prepare body fluid slides either on resuspended sediment after centrifugation, or with a drop of undiluted or RBC-lysed hypercellular fluid, using a slide-on-slide method. The slide-on-slide technique involves the following steps:²

1. Place a drop of the sample in the middle of a glass slide.
2. Place a second slide over the first, perpendicular or parallel, and allow the sample to spread by capillary action. Do not apply any pressure.
3. Pull the slides straight apart laterally in one smooth motion. Do not pull up or down as the slides are being separated.
4. Allow the slides to air dry.

CRITICAL THINKING QUESTION

30-1 If there are no cells seen in the hemacytometer during the manual cell count, why is it important to prepare a cytocentrifugation slide?

See answers to all Critical Thinking Questions at the back of this book.

Cellular Components of Body Fluids

Under normal conditions, cells present in body fluids are composed of peripheral blood leukocytes that are capable of migration through the vascular endothelium into extravascular spaces and a small number of epithelial cells sloughed from the membrane that lines the compartment. The most common types of cells encountered are neutrophils, lymphocytes, macrophages, and tissue cells. Other cells that are less frequently seen are eosinophils, basophils, and mast cells.

Neutrophils

Neutrophils are frequently observed in pleural, peritoneal, and pericardial fluids, as well as synovial fluids, but normally constitute fewer than 25% of the total cells in the differential analysis. Very few, if any, should be observed in CSF. After staining, the neutrophils would appear as in a peripheral blood smear; however, the process of cytocentrifugation may cause the nuclei to be pushed to the cell's periphery, resulting in an artifactual hypersegmented appearance (see Fig. 30-1). Some cells can be degenerated, exhibiting cytoplasmic vacuolization and nuclear pyknosis.

Immature neutrophils (i.e., promyelocytes, myelocytes, and metamyelocytes) are not commonly seen; if present, they may represent a chronic inflammatory process or a bone marrow disorder (e.g., myeloproliferative, myelodysplastic, or leukemic syndrome). Blasts are found only in the presence of bone marrow disorders.

Lymphocytes

Lymphocytes are often seen in all types of fluids in variable numbers. They can vary in appearance from small to large and are normally reactive in their nuclear morphology. The nuclei are artifactually more prominent in cytocentrifuge preparations. The shape of the nucleus might be irregular, and the cytoplasm can have artifactual projections (see Figs. 30-2 and 30-3).⁴ Lymphocyte morphology in malignancy is determined by the type of neoplasm involved (i.e., leukemia or lymphoma). The lymphocytes seen in these conditions will appear homogeneous, as they are clonal in origin. Plasma cells, if present, are usually seen only in chronic inflammatory disorders.

Macrophages

Macrophages, or phagocytes, are transformed monocytes, having migrated from the peripheral blood to the tissues. Macrophages present in body fluids can appear similar to monocytes seen in peripheral blood smears or may be larger with abundant, vacuolated cytoplasm (Figs. 30-4 and 30-5). Macrophages without phagocytosed material are referred to as histiocytes. Classifying these cells between types is not clinically important. In some cases, however, phagocytosed organisms or cells may be of diagnostic importance. Some macrophage cells' vacuoles may fuse together into one large cytoplasmic vacuole, causing the nucleus to become flattened against the cell membrane; this gives the cell the appearance of a **signet ring** (Fig. 30-6).

Tissue Cells

Benign tissue cells are seen in all fluids and must be differentiated from malignant cells. Serous fluids contain benign mesothelial cells, as they are normally sloughed from the membrane. These appear as large cells with moderate to abundant cytoplasm that may be stained a light or dark blue and may contain phagocytosed debris or granules. The nucleus is eccentric with a smooth nuclear outline and a fine, homogeneous chromatin pattern. Nucleoli may be large and prominent, usually uniform in size and shape (Figs. 30-7 and 30-8). The appearance of mesothelial cells can vary within the same fluid specimen, which may cause some difficulty in identification (Fig. 30-9).

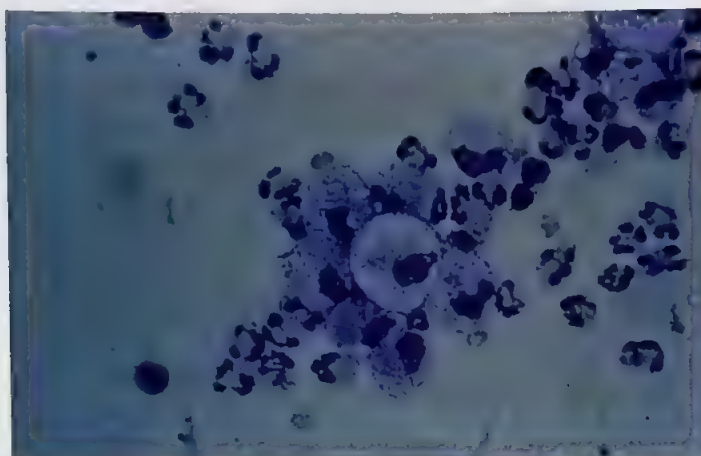


FIGURE 30-4 Histiocytes/macrophages (center). Also, monocytes and neutrophils. Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

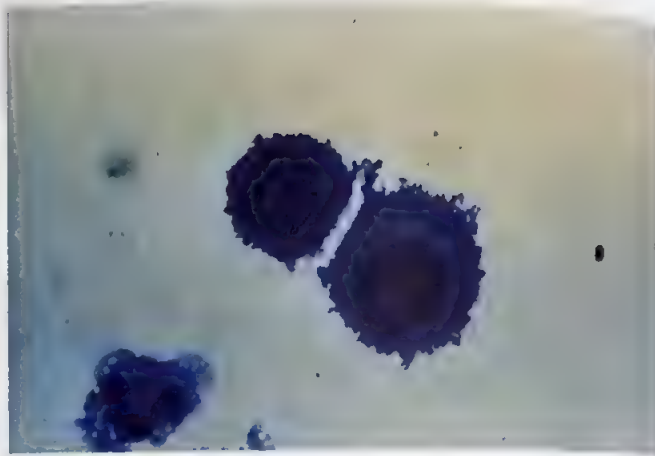


FIGURE 30-7 Mesothelial cells. Normal, quiescent. Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

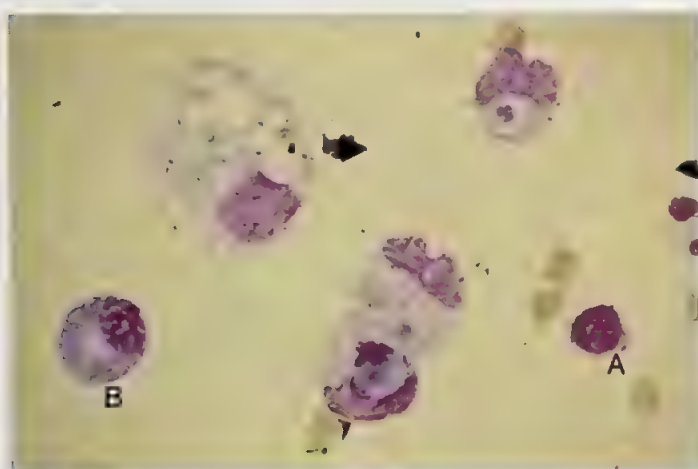


FIGURE 30-5 Macrophages. Also, a lymphocyte (A) and a plasma cell (B). Wright stain, $\times 1,000$ magnification. (Courtesy of Judith Brody, M.D., Northwell Health Laboratories, Lake Success, New York.)

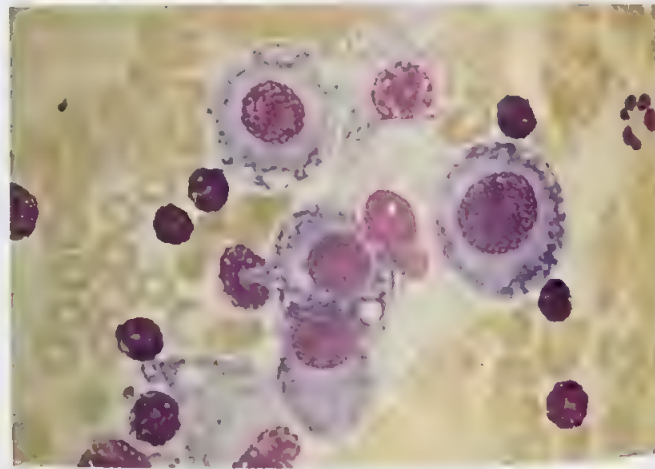


FIGURE 30-8 Mesothelial cells; degenerated with peripheral vacuoles, resembling macrophages. Wright stain, $\times 1,000$ magnification. (Courtesy of Judith Brody, M.D., Northwell Health Laboratories, Lake Success, New York.)

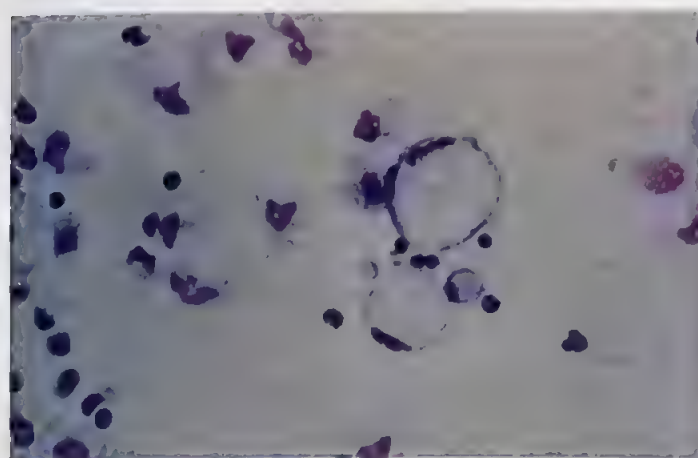


FIGURE 30-6 Signet-ring type macrophages. Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

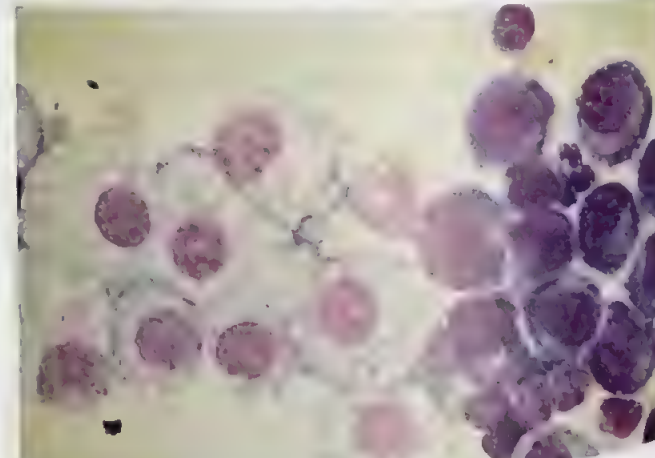


FIGURE 30-9 Macrophages (right) and mesothelial cells (left). Wright stain, $\times 1,000$ magnification. (Courtesy of Judith Brody, M.D., Northwell Health Laboratories, Lake Success, New York.)

CSF tissue cells (choroid plexus cells, ependymal cells) tend to form clusters. They may have cytoplasmic granules and slightly irregular nuclei. Arachnoid mater cells are frequently seen as a **syncytium**: a mass of cytoplasm containing several nuclei. These benign cells are usually only seen in the

CSF from infants or adults who have had recent neurosurgery or an implanted reservoir to deliver chemotherapeutic agents or antimicrobials directly into the CNS.⁴ Synovial cells have a similar appearance to mesothelial cells, although the cytoplasm appears denser than the mesothelial

cell. Cartilage cells (chondrocytes) may be seen in a variety of arthritic conditions. These cells contain a central, small, round, pyknotic nucleus surrounded by a clear zone and a distinct burgundy-colored cytoplasm.⁴

Eosinophils, Basophils, and Mast Cells

Eosinophils, basophils, or mast cells may be present in small numbers in body fluids. Increased numbers can be seen in various disorders, and their presence may or may not be correlated with their concentration in the peripheral blood. Mast cells can be distinguished from basophils by having a round nucleus (not segmented) and a higher number of cytoplasmic granules that are smaller in size than those usually seen in basophils.⁴

Serous Fluids: Pleural, Pericardial, and Peritoneal

Serous fluids are discussed together because they present similar findings in normal and pathological states. Normally, these compartments contain a minimal amount of fluid, only enough to keep the membranes moist and slippery. Fluid is produced by the parietal lining by plasma filtration through capillary endothelial cells and absorbed by the visceral lining. Causative factors for effusions are listed in Box 30-3. Pathology affecting any one or more of these factors, as well as trauma, infection, and malignancy, can result in an abnormal fluid collection, known as an effusion.

Effusions: Transudates and Exudates

Effusions that accumulate due to a systemic disease state are categorized as **transudates**. Effusions that accumulate due to a primary pathological state within the compartment are categorized as **exudates**. The purpose of identifying the type of effusion as transudative or exudative is to determine whether further laboratory or other extensive diagnostic tests are required to identify the cause. Transudates usually do not require further laboratory or diagnostic evaluations. Table 30-1 compares the features of transudates and exudates.

Occasionally, differentiation between transudates and exudates is not possible based upon the WBC, specific gravity, total protein (TP), and fluid TP. In this case, the analysis of fluid lactate dehydrogenase (LDH), glucose, and cholesterol can assist in the identification by comparison to a simultaneously collected serum specimen.^{5,6}

Exudates tend to be cloudy, turbid, or purulent due to the presence of lipids or the increased WBC count, and frequently clot on standing due to the presence of fibrinogen. If a portion

TABLE 30-1 Comparison of Transudates and Exudates

Feature	Transudates	Exudates
Cause	Form as a result of increased capillary hydrostatic pressure, as seen in congestive heart failure, or decreased plasma oncotic pressure, as seen in the hypoproteinemia of the nephrotic syndrome or liver failure	Form as a result of increased capillary permeability and/or decreased lymphatic resorption
Appearance	<ul style="list-style-type: none"> • Clear, pale yellow • Do not clot 	<ul style="list-style-type: none"> • Cloudy, turbid, purulent, bloody • Clots on standing
WBC	<1,000 cells/ μ L	>1,000 cells/ μ L
Specific gravity	\leq 1.015 g/dL	>1.015 g/dL
Total protein	\leq 3.0 g/dL	>3.0 g/dL
Fluid/serum TP ratio	<0.5	>0.5
LDH	<200 IU/L	>200 IU/L
Fluid/serum LDH ratio	LDH ratio: <0.6	>0.6
Glucose	\sim Serum/plasma glucose	<Serum/plasma glucose
Cholesterol	<60 mg/dL	>60 mg/dL
Fluid/serum cholesterol ratio	<0.3	>0.3

of the sample is centrifuged, a clear supernatant indicates the presence of an abundance of leukocytes or cellular debris; a white supernatant is caused by the presence of lipids.

If the fluid has a milky or opaque appearance that remains in the supernatant after centrifugation, this is known as a **chylous effusion**: an exudate resulting from leakage or a blockage of the lymphatic vessels. It is rich in chylomicrons, has an elevated triglyceride level (greater than 110 mg/dL), and contains predominantly lymphocytes. Chylous effusions most often result from a malignancy such as lymphoma or carcinoma, or from trauma.

An exudate unrelated to the lymphatics, known as a **pseudochylous effusion**, results from a persistent, chronic effusion due to diseases such as tuberculosis (TB) and RA-associated inflammation of the pleura (i.e., rheumatoid pleuritis). Unlike a chylous effusion, this exudate is caused by the breakdown of cellular lipids, does not contain chylomicrons, and usually has a low triglyceride level (less than 50 mg/dL). Its appearance is caused by cellular debris and cholesterol crystals, and it contains a mixed reactive cell population with many inflammatory and necrotic cells. Table 30-2 compares chylous and pseudochylous effusions.

BOX 30-3 Causative Factors for Effusions*

1. Increased capillary hydrostatic pressure
2. Increased capillary permeability
3. Decreased lymphatic resorption
4. Decreased plasma oncotic pressure

*A defect in one or more of these factors results in the production of an effusion.

TABLE 30-2 Comparison of Chylous and Pseudochylous Effusions

Feature	Chylous Effusion	Pseudochylous Effusion
Appearance	Milky-white; opaque; supernatant remains milky; forms creamy top layer upon standing	Milky-white, yellow, green; opaque; supernatant remains milky
Cause	Leakage, damage, or blockage of thoracic duct	Chronic effusion; breakdown of cellular lipids
Disease process	Trauma, malignancy	TB, Rheumatoid pleuritis
Presence of chylomicrons	Rich in chylomicrons	Chylomicrons absent
Triglycerides	Increased (>110 mg/dL)	Decreased (<50 mg/dL)
Cellular content	Lymphocytosis	Mixed reactive cell population; necrotic cells, cholesterol crystals

Trauma, aneurysm, malignancy, pancreatitis, or TB may also produce a bloody or hemorrhagic effusion.

The previously described analyses are not always able to definitively distinguish transudates from exudates when the results are equivocal; the patient's clinical history and physical examination can provide further information. The finding of an exudate must always be followed by more extensive tests to determine its cause.

Cellular Responses, Microorganisms, and Malignant Cells

Effusions can occur due to a wide variety of local or systemic disease processes. They may be categorized as normal cellular, benign/reactive, or malignant based on the present cell types. Normal cellular effusions, as their name implies, are neither reactive nor malignant. Cellular morphology is normal regardless of the cause of the effusion. A serous fluid is categorized as benign/reactive when cellular abnormalities are caused by infection, inflammation, or other sterile reactive processes (e.g., pulmonary infarction or cirrhosis of the liver). Serous fluids associated with bacterial infections will demonstrate an inflammatory cellular response that consists predominantly of segmented neutrophils on differential analysis. These cells may reveal Döhle bodies, toxic granulation, and/or vacuolization. Viral, fungal, or mycobacterial infections may be associated with a predominance of lymphocytes

or show a mixed inflammatory response. These lymphocytes are frequently reactive and transformed, resembling immunoblasts. Benign/reactive lymphocytes consist of a heterogeneous population of cells with varying nuclear shape, amount of cytoplasm, and degree of cytoplasmic basophilia, whereas malignant lymphoma cells would appear as a homogeneous population, most with the same nuclear and cytoplasmic features. Eosinophilic responses can occur with parasitic or fungal infections, hypersensitivities, pneumothorax, some immune disorders, and peritoneal dialysis. Many will have an inflammatory cell response that is not diagnostic for any specific disorder; these would be referred to as nonspecific, reactive cellular responses. Table 30-3 outlines the reactive processes and cell types present in serous effusions.

ADVANCED CONTENT

In rare cases, lupus erythematosus (LE) cells form spontaneously. An LE cell is a phagocyte; either a neutrophil or monocyte that has ingested a naked nucleus showing a homogeneous, smooth chromatin pattern. This finding is suspicious but not diagnostic of SLE; other autoimmune disorders may also show this phenomenon.

TABLE 30-3 Reactive Processes and Cell Types Present in Serous Effusions

Reactive Process	Cell Types Present
Acute bacterial infection (e.g., pneumonia; peritonitis; pericarditis)	<ul style="list-style-type: none"> Greater than 50% neutrophils Many macrophages
Chronic inflammation or infection (e.g., rheumatoid arthritis and tuberculosis)	<ul style="list-style-type: none"> Many reactive mesothelial cells, macrophages, and lymphocytes Few plasma cells and neutrophils
Chronic sterile reactive processes (e.g., cirrhosis; pulmonary embolism/infarction)	<ul style="list-style-type: none"> Many reactive mesothelial cells and macrophages Few neutrophils and lymphocytes Signet ring-type macrophages can be present
Eosinophilic effusions (e.g., parasitic/fungal infections; hypersensitivities; pneumothorax; immune disorders; peritoneal dialysis)	<ul style="list-style-type: none"> Greater than 10% eosinophils

Mesothelial cells, as previously described, can have a wide variation in morphology. They may show nonspecific reactive changes that include multinuclearity, the presence of nucleoli, mitotic activity, and sometimes an increase in cell size (Figs. 30-10 and 30-11). They have been classified into categories based on their diverse morphology as quiescent, hypertrophied, epithelioid, phagocytic, and senescent.⁷

Quiescent mesothelial cells are the normal constituents of the membrane that are shed into the fluid (see Fig. 30-7). Hypertrophied cells are larger, with more prominent nucleoli, and can be seen in reactive processes that cause cellular hyperplasia (Fig. 30-12). Epithelioid mesothelial cells are seen as clumps or sheets resulting from the disrupted membrane surfaces in reactive processes (Fig. 30-13). Phagocytic mesothelial cells are indistinguishable from macrophages or histiocytes (Fig. 30-14). The senescent mesothelial cell is degenerated, with a single large vacuole forming a signet ring configuration (see Fig. 30-15). The nucleus may show

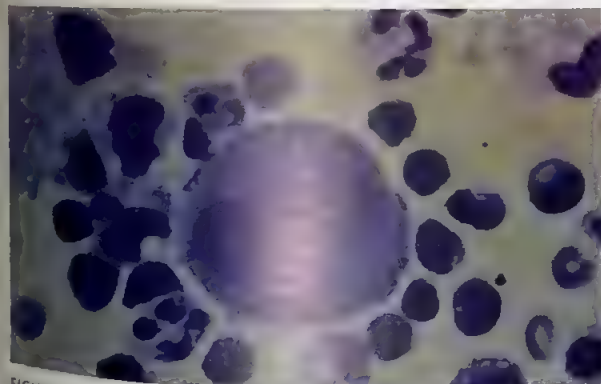


FIGURE 30-10 Reactive mesothelial cells; some binucleated. Also, macrophages, neutrophils, and lymphocytes. Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

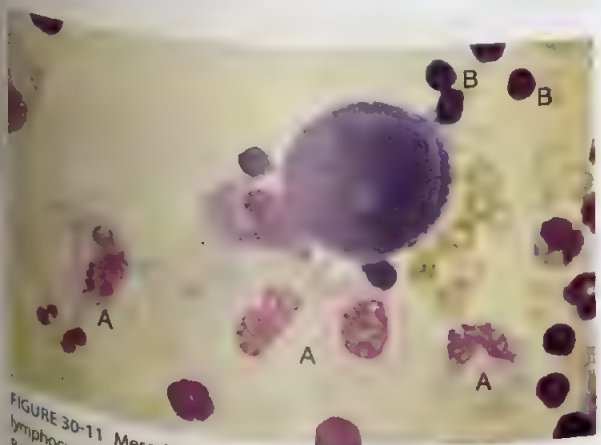


FIGURE 30-11 Mesothelial cell; multinucleated. Also, macrophages (A) and lymphocytes (B). Wright stain, $\times 1,000$ magnification. (Courtesy of Judith Brody, M.D., Northwell Health Laboratories, Lake Success, New York.)

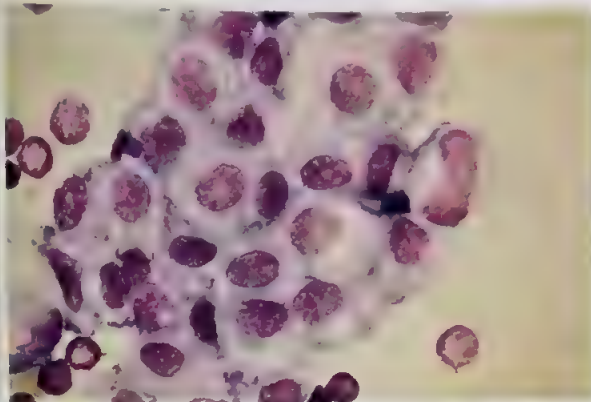


FIGURE 30-12 Hyperplastic mesothelial cells. Wright stain, $\times 400$ magnification. (Courtesy of Judith Brody, M.D., Northwell Health Laboratories, Lake Success, New York.)

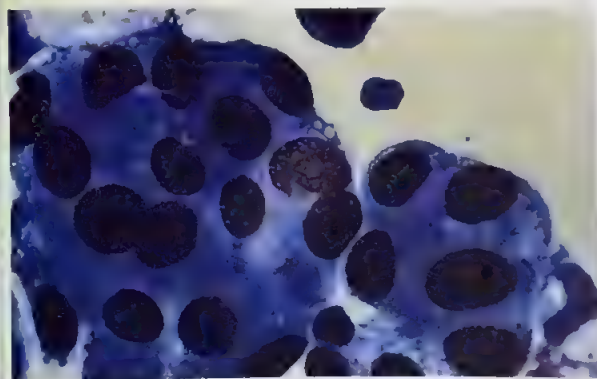


FIGURE 30-13 Reactive mesothelial cells in sheets (epithelioid) with prominent nucleoli. Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

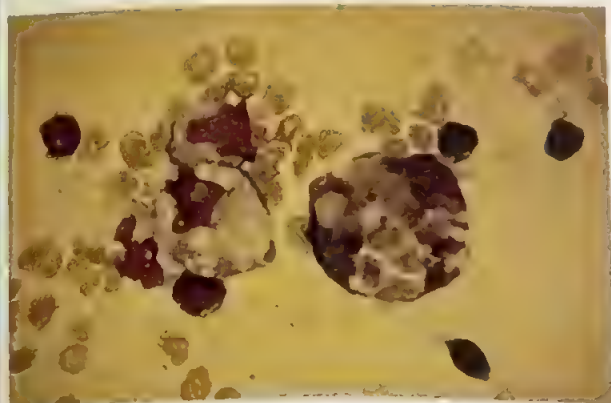


FIGURE 30-14 Phagocytic macrophage/mesothelial cell. Note the ingested RBCs (left). Wright stain, $\times 1,000$ magnification. (Courtesy of Judith Brody, M.D., Northwell Health Laboratories, Lake Success, New York.)

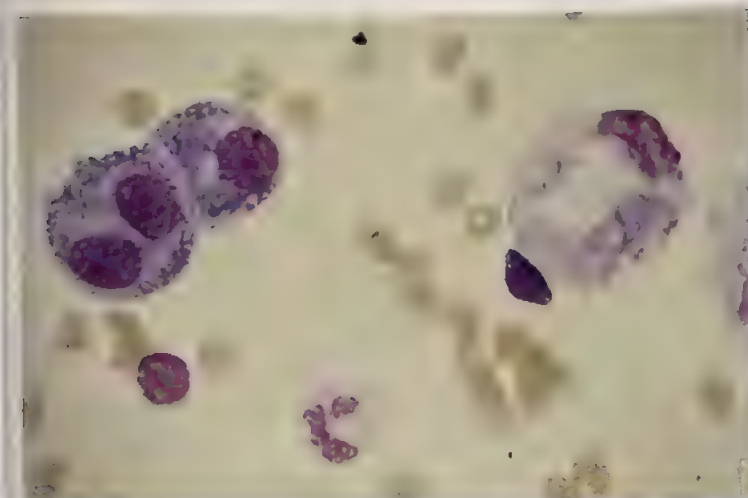


FIGURE 30-15 Senescent mesothelial cell (right); becoming signet ring configuration. Wright stain, $\times 1,000$ magnification. (Courtesy of Judith Brody, M.D., Northwell Health Laboratories, Lake Success, New York.)

pyknosis and karyorrhexis: one or more spherical, densely staining nuclear fragments. All, except quiescent mesothelial cells, can resemble malignant cells. Extreme caution must be taken to avoid misinterpretation; referral to pathology and/or cytology is required.

Most types of pathogenic bacterial and fungal organisms will stain with the Wright (Wright-Giemsa) stain and are detectable on a routine cytocentrifuge preparation. Bacteria will stain blue regardless of the Gram's stain reaction (Fig. 30-16). If the organisms are intracellular (Fig. 30-17), the presence of infection is indicated as opposed to in vitro contamination of the slide or stain. Most pathogenic yeasts are found in CSF as opposed to serous fluids. They may or may not be found intracellularly. The most frequently encountered fungal organisms in fluids are:

- *Cryptococcus* spp.
- *Histoplasma* spp.
- *Candida albicans*
- *Candida tropicalis*

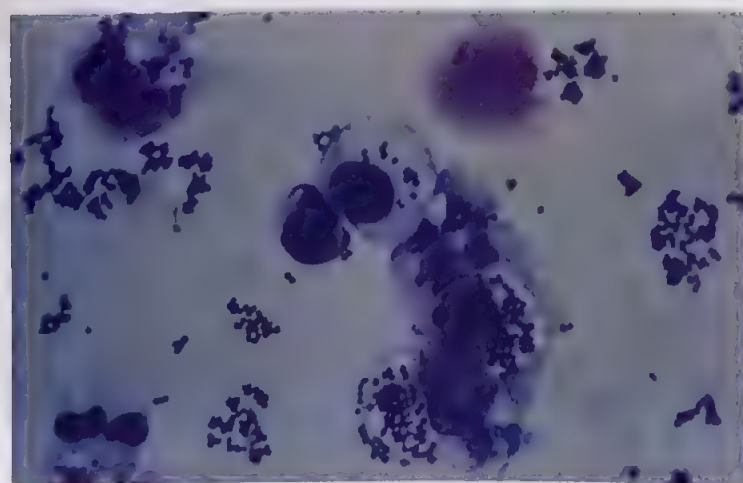


FIGURE 30-16 Bacteria in body fluid: *Staphylococcus* species; note the intra- and extracellular distribution. Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

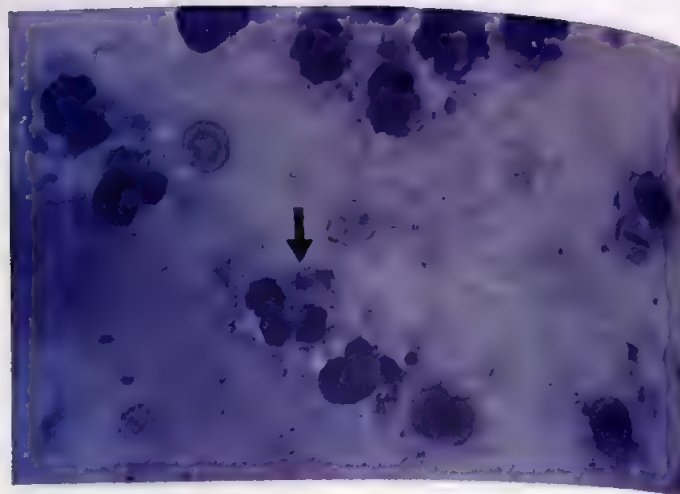


FIGURE 30-17 Bacteria in body fluid: *Neisseria* species; note the intracellular diplococci in neutrophils. Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

Malignancy is a major cause of serous effusion; therefore, serous fluids may contain malignant cells from a variety of neoplasms. Their identification is critical for accurate diagnosis. A malignancy may already be known to exist, but its presence in the fluid demonstrates metastasis; it may also be an initial diagnosis. The laboratory professional must look at the entire cellular area of the slide under low power to detect suspicious clusters of cells. It is not possible to distinguish malignant cells from benign reactive cells by a single cellular characteristic; the cells must be judged based on collective criteria that include the gross appearance of the fluid as well as the cellular features. Additional studies such as immunocytochemistry, flow cytometry, tumor marker analyses, and biochemical and enzyme assays may also be required.

The general features of malignant cells are:

- Multilayered formations; three-dimensional spheroidal arrangements (ball-like formations) (Figs. 30-18 and 30-19)
- Large cell size (often greater than $50\ \mu\text{m}$ diameter) or giantism
- An irregular nuclear membrane; jagged or showing multiple folds

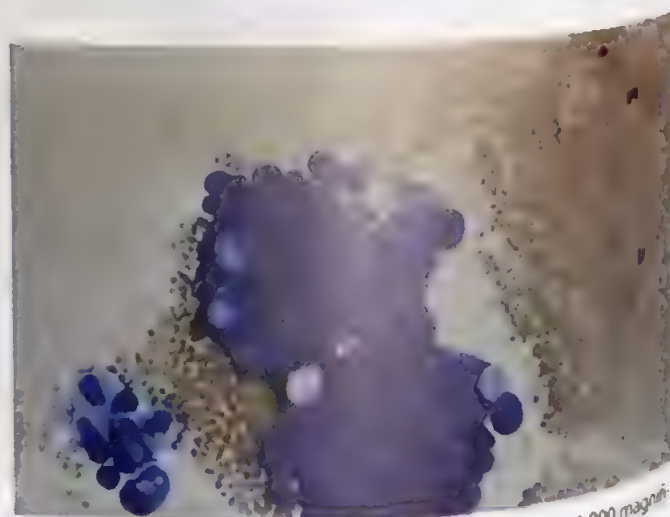


FIGURE 30-18 Clusters of malignant cells. Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

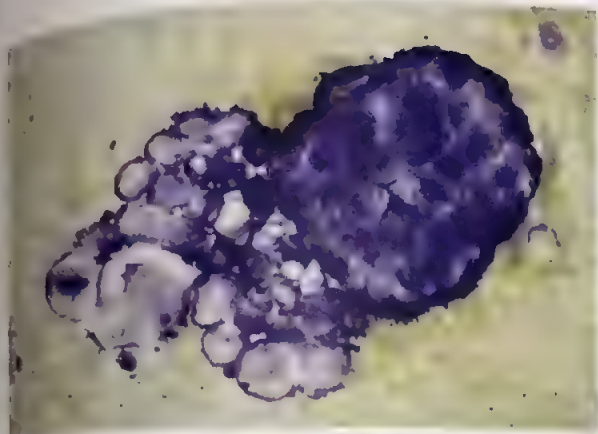


FIGURE 30-19 Cell-ball formation; malignant cell cluster. Wright stain, $\times 500$ magnification. (Courtesy of Judith Brody, M.D., Northwell Health Laboratories, Lake Success, New York.)

- Multinucleation; nuclear molding; Single file arrangements (Figs. 30-20 and 30-21)
- Nuclear hyperchromasia; unevenly distributed chromatin
- Prominent nucleoli with irregular size and shape
- High/variable N:C ratio
- Increased/abnormal mitotic activity (Fig. 30-22)
- Bizarre vacuolation; abnormal inclusions (Fig. 30-23)
- Cannibalism (one cell engulfing/ingesting another) (Fig. 30-24)
- Uneven staining of cytoplasm

Two very characteristic features of malignant cells in fluid preparations are **nuclear molding** and **paranuclear blue body inclusions**. Nuclear molding describes the process whereby the nucleus of one cell molds around the shape of an adjacent cell. This occurs with the cohesive growth of tumor cells that require the presence of tight junctions between the cytoplasmic membranes of the cells. It is most often seen in small cell carcinoma, but it can be seen in any type of carcinoma. Paranuclear blue body inclusions occur in small cell carcinoma or rarely in sarcoma. They may represent early cell degeneration or phagocytosed material. The

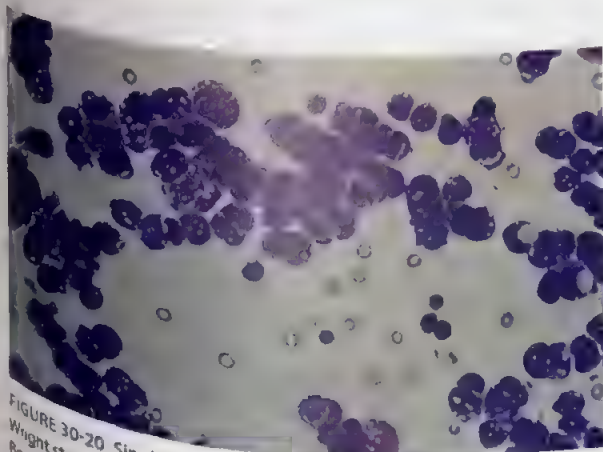


FIGURE 30-20 Single file cellular arrangements: Breast carcinoma. Wright stain, $\times 500$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

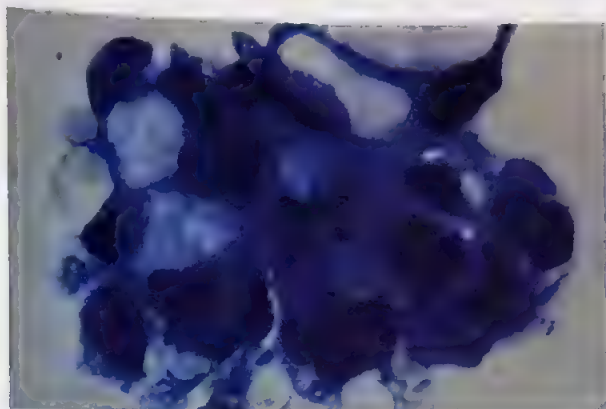


FIGURE 30-21 Nuclear molding. Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

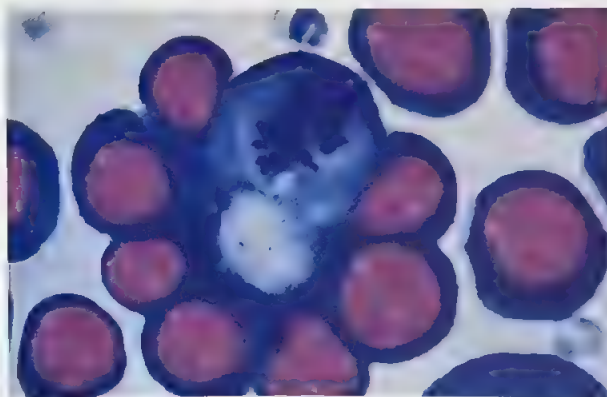


FIGURE 30-22 Abnormal mitotic activity; acinar (glandular) arrangement of malignant cells. Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

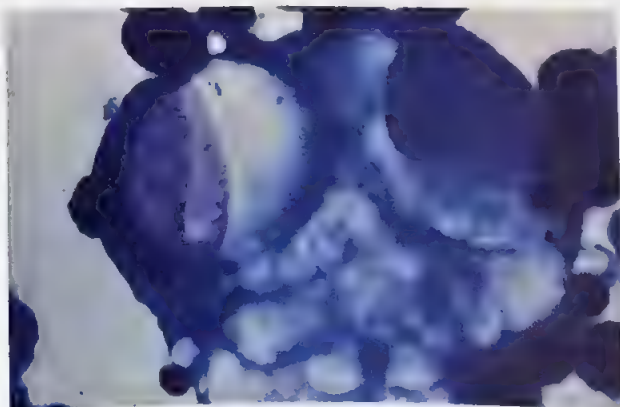


FIGURE 30-23 Foamy, bizarre vacuolization in cytoplasm of malignant cells. Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

inclusions may appear to be intranuclear depending on the orientation of the cell on the slide. They are only seen on the Wright (Wright-Giemsa)-stained preparations. Cytology preparations and other laboratory studies are required to specifically identify the type of malignancy (Table 30-4).

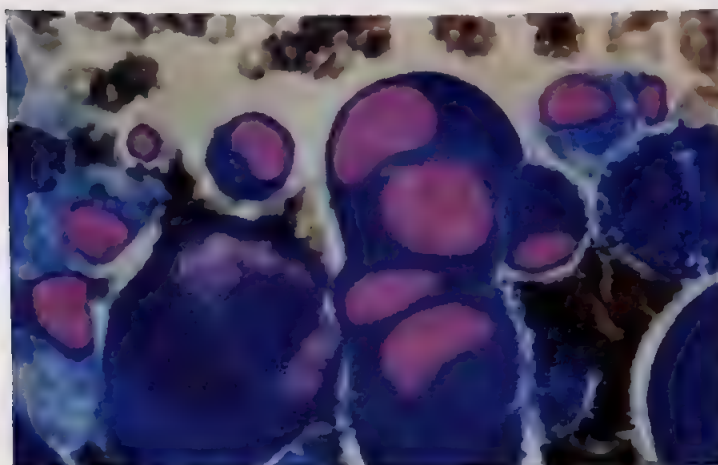


FIGURE 30-24 Nuclear molding; cannibalism of malignant cell (left). Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

TABLE 30-4 Features of Nonhematologic Malignant Cells in Serous and Cerebrospinal Fluid

Feature	Description
Size	<ul style="list-style-type: none"> Large, frequently $>50\ \mu\text{m}$ Giantism High/variable N:C ratio ($\geq 1:1$)
Nuclear	<ul style="list-style-type: none"> Multinucleation; variable size Pleomorphic, nonuniformity Irregular nuclear membrane; herniation into cytoplasm; clefts Nuclear hyperchromasia Unevenly distributed chromatin Nuclear molding Prominent nucleoli with irregular size and shape Increased/abnormal mitotic activity
Cytoplasmic	<ul style="list-style-type: none"> Uneven staining of cytoplasm Bizarre vacuolization or large, singular vacuole Abnormal inclusions Fused cytoplasmic membranes Cannibalism
Cellular Arrangement	<ul style="list-style-type: none"> Multilayered formations 3-D forms Spherical aggregates Cell clumps lacking, or having poorly defined margins Wraparound, rosette or acinar formations Single cells

Hematopoietic malignancies that can most commonly be found in body fluids are:

- Lymphocytic and nonlymphocytic leukemia
- Non-Hodgkin lymphomas
- Hodgkin's lymphoma
- Plasma cell neoplasms

The abnormal cells found in a fluid in any of these disorders have the same morphological features as seen in the peripheral blood and bone marrow.

The most common nonhematopoietic malignancies that can be seen in body fluids are:

- Small cell carcinoma (oat cell)
- Metastatic adenocarcinoma (breast, ovarian, stomach, colon, rectum)

Types of Effusions, Laboratory Analysis, and Clinical Correlations

As previously noted, effusions are an abnormal accumulation of fluid, and the location of the effusion determines the necessary terminology, collection procedure, analysis, and correlations (Table 30-5).

Pleural and Pericardial Effusions

A **pleural effusion** is defined as an abnormal accumulation of fluid in the pleural cavity. It occurs when the rate of production exceeds the rate of absorption. Conditions that affect capillary pressure or permeability, colloid osmotic pressure, lymphatic drainage, or increased negative intrapleural pressure (which can occur with **atelectasis** [collapsed lung]) can lead to the formation of a pleural effusion. The presence of large amounts of fluid may restrict lung expansion, causing dyspnea and mild hypoxemia. Pleural effusions caused by congestive heart failure, nephrotic syndrome, or cirrhosis tend to be bilateral, whereas unilateral effusions are caused by diseases that develop below the diaphragm, such as cirrhosis with ascites, hepatic abscess, pancreatitis, and tumors.⁴

Specimen Collection and Processing

Analysis of a pleural effusion begins with the removal of fluid by a **thoracentesis** (see Table 30-5). Portions of the pleural (or thoracentesis) fluid are placed into heparinized tubes for chemical analysis, sterile tubes for microbiological cultures and Gram and/or acid-fast stains, and tubes containing ethylenediaminetetraacetic acid (EDTA) for cell counts and differential analysis. Additional nonadditive evacuated tubes may also be collected for cytology and immunophenotyping studies. Thoracentesis may also be used for therapeutic purposes.

The gross appearance of the pleural fluid provides important diagnostic clues to the nature of the disease process. Pure blood in the pleural cavity, a **hemothorax**, can result from severe chest injuries, stab or gunshot wounds, or surgical procedures.

TABLE 30-5 Body Fluid and Collection Procedure Nomenclature

Body Cavity/Region	Fluid Name	Collection Procedure
Pleural cavity/lungs	Pleural (serous)	Thoracentesis
Pericardial cavity/heart	Pericardial (serous)	Pericardiocentesis
Peritoneal cavity/abdomen	Peritoneal, ascites (serous)	Paracentesis
CNS; subarachnoid space/brain and spinal cord	Cerebrospinal	Lumbar puncture, "spinal tap"
Synovial cavity/joints	Synovial	Arthrocentesis

bloody or hemorrhagic effusion, in the absence of trauma, almost always suggests the presence of malignancy or occasionally a pulmonary infarction. Leakage of the thoracic duct causes a **chylothorax**, most commonly caused by a malignancy such as lymphoma or carcinoma.^{8,9} This produces a milky-white, opaque pleural fluid that remains opaque after centrifugation. Infection of the pleural space by bacterial pneumonia or a ruptured lung abscess will cause **empyema**: the collection of pus in the pleural cavity. This fluid will be turbid to opaque and have an extremely elevated WBC count of 25,000/ μ L or higher. After centrifugation, the supernatant of this type of fluid will be clear due to the removal of the cellular debris.

The abnormal accumulation of fluid in the pericardial space is known as a **pericardial effusion**. This is most frequently caused by damage to the lining of the cavity and increased capillary permeability. The function of the normal pericardium is to oppose dilation of the heart; this is reflected by the mean central venous pressure. Interference with pericardial venous and lymphatic drainage, which occurs in acute pericarditis, can lead to the formation of an effusion. The volume of fluid, the rate of its formation, and the elasticity of the pericardial membrane will determine what effect the effusion will have on cardiac function. A small effusion may have no effect and not produce any symptoms; a large effusion that develops very slowly may also not cause any symptoms, provided that the pericardium has the ability to stretch. A severe complication of pericarditis or a traumatic injury can cause a condition known as **cardiac tamponade**. This occurs when pericardial fluid or blood within the pericardial space, under increased pressure, restricts the motion of the heart. This produces a state of critical cardiovascular dysfunction, causing decreased cardiac output and hypotension, and if left untreated will cause death. Treatment requires the aspiration of the pericardial fluid in a procedure known as **pericardiocentesis** (see Table 30-5). A portion of the aspirate is sent to the laboratory for analysis for pericardiocentesis, as discussed earlier. A wide variety of diseases and disorders can produce pericardial effusions such as neoplastic disease, infectious agents, cardiovascular disease, renal disease, collagen vascular diseases, and hemorrhagic events (e.g., trauma, aneurysm, or anticoagulant therapy).

Laboratory Analysis and Clinical Correlations

Qualitative Analysis The hematology laboratory will receive the fluid in tubes containing EDTA, which anticoagulates and preserves the cell morphology for analysis. If the specimen was not properly collected or handled and it arrives in the laboratory completely clotted, the analysis cannot be performed. If the specimen is partially clotted, which can occur if the specimen is not properly mixed after collection, the cell count would be inaccurate, but an attempt should be made at performing the morphological examination for the presence of reactive or malignant cells, with the required comment on the report and prompt physician notification.

Gross visual inspection of the fluid is initially performed. A normal pleural fluid is pale yellow in color and transparent. Abnormal findings include cloudy, turbid, bloody, yellow, green, or chylous. If the fluid is grossly bloody and a microhematocrit performed on the specimen has a packed cell volume

(PCV) greater than 50% of the peripheral blood PCV, this would indicate the presence of a hemothorax.

A normal pericardial fluid is also pale yellow in color and transparent. Turbidity can be caused by infection or malignancy. Grossly bloody fluid may result from trauma or disease, or the possibility that intracardiac blood was aspirated into the specimen during pericardiocentesis. The findings of a chylous fluid are rare, but it can be caused by the leakage of lymphatic vessels from trauma, lymphoma, or carcinoma.

Quantitative Microscopic Analysis Next, the specimen is examined for its cellular concentration. RBC and TNC counts are performed on the undiluted fluid. Cloudy or turbid fluids may be diluted with saline or buffered cell diluent for manual counting, and the cell count multiplied by the appropriate dilution factor. Automated analyzers can provide RBC, TNC, and WBC counts. Cell counts are of limited value in making a differential diagnosis because they are nonspecific. As few as 5,000 RBCs/ μ L will cause a blood-tinged appearance to the fluid. Red blood cell counts greater than 100,000/ μ L in pleural fluid are highly suggestive of malignant neoplasm, trauma, pulmonary embolism, or infarction.⁹ The performance of a microhematocrit can be useful in distinguishing a hemorrhagic effusion from aspirated blood in the specimen. Generally, a WBC count less than 1,000/ μ L is associated with a transudate, while higher counts are associated with exudates and suggestive of microbial infection or malignancy. These values alone should not be used to classify the fluid as transudative or exudative; other biochemical analyses must be considered, as previously discussed.

Examination of the Wright (Wright-Giemsa)-stained cytocentrifuge preparation is required regardless of the cell count. Concentration of the fluid will demonstrate the presence of cellular material. The entire circular field of stained sediment is first scanned under the low-power objective (10 \times), searching for evidence of cellular clumps or sheets, and the presence of large cells. A standard 100-cell differential is then performed under high-power oil immersion (50 \times or 100 \times objective). If fewer than 100 cells are present, all the cells in the preparation are counted; the actual numbers of cells counted and the percentages of each cell type are calculated and reported. The cells are categorized in the same manner as peripheral blood differential counts, with the addition of mononuclear cell categories that are used for macrophages, mesothelial cells, and malignant cells. These cell types are counted and reported as a percentage, with a comment describing the types of cells present. This method of counting has the advantages of (1) cells that are difficult to identify morphologically may be grouped together and (2) no attempt is made to count nonhematologic malignant cells individually because they often occur in clumps and are very difficult to count. Malignant hematologic (leukemia, lymphoma) cells are counted and reported as in peripheral blood differentials. All stained fluid slides with atypical, suspicious, or malignant cells must be reviewed by a pathologist before reporting.

Morphological Analysis Segmented neutrophils in pleural or pericardial fluid appear morphologically identical to those in the peripheral blood, although cytocentrifugation may introduce artifactual changes, as previously discussed. Immature neutrophils are rarely seen except in chronic myelogenous

leukemia or leukoerythroblastic conditions. Neutrophils in chronic effusions can show signs of cellular degeneration, with vacuolization and decreased granularity in the cytoplasm. They can also exhibit pyknosis or karyorrhexis and be mistaken for nucleated red blood cells (NRBCs) (Fig. 30-25). Neutrophils are present in higher concentrations in exudates of a bacterial etiology, such as a pleural effusion of bacterial pneumonia or a ruptured lung abscess. In pericardial fluid, a predominance of neutrophils is seen in bacterial pericarditis (see Table 30-3).

Lymphocytes in pleural fluid resemble small peripheral blood lymphocytes and are seen in variable numbers. They may be variable in size and have an immature or reactive (transformed) appearance in response to various stimuli. The nucleus can be cleaved and exhibit nucleoli that are often more prominent than those in peripheral blood lymphocytes. Some of these nuclear changes are artifactual effects of cytocentrifugation (see Fig. 30-2). Degenerative changes can occur in aged specimens. Increased numbers of lymphocytes (lymphocytosis) are seen in effusions from patients with tuberculosis (TB), malignancy, and viral infections (see Table 30-3).

When present, plasma cells resemble those encountered in the bone marrow. Increased numbers can accompany the increase in lymphocytes that is seen in patients with multiple myeloma or other disorders with associated lymphocytosis (Fig. 30-26). They are also seen in effusions from patients with TB, RA, malignancy, Hodgkin's disease, and so forth. If a malignancy is suspected, referral to pathology is mandatory and cytological examination must be performed.

Mononuclear phagocytes (histiocytes or macrophages) are seen in variable numbers in both benign and malignant effusions. The number of mononuclear cells increases as the inflammatory process becomes chronic. *In vivo*, LE cell formation has been observed as well. If the fluid remains at room temperature, *in vitro* LE cell formation can occur.

Pleural fluid eosinophilia (eosinophils seen in increased numbers greater than 10%) is a nonspecific finding, commonly seen in idiopathic effusions. It may signify that air

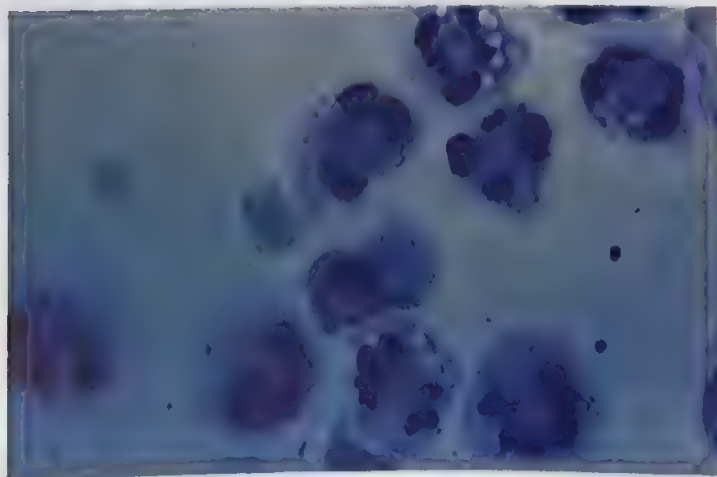


FIGURE 30-25 Neutrophils with vacuolization, fragmentation, decreased granulation; one pyknotic neutrophil (center). Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

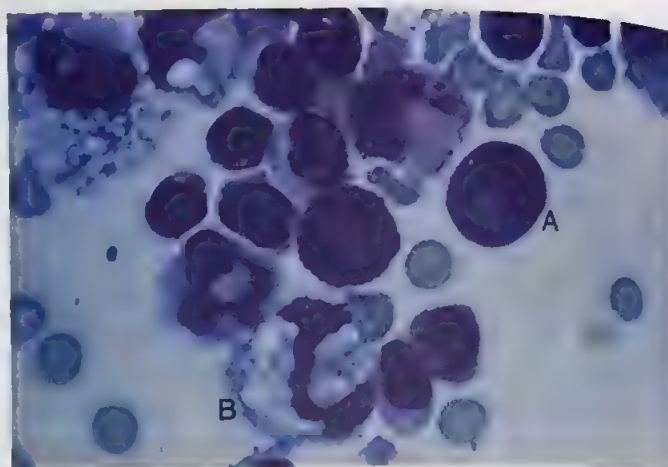


FIGURE 30-26 One plasma cell (A), one plasmacytoid lymphocyte, reactive and normal lymphocytes in a chronic effusion; note the monocyte/macrophages (B). Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

(pneumothorax) or blood (hemothorax) has been introduced into the pleural space. By itself, it is not diagnostically significant, but it may be manifested in parasitic or fungal diseases, malignancy, connective tissue disorders, hypersensitivities, or pulmonary infarction. The presence of eosinophilia in an exudative effusion is an indication that the condition is probably not malignant or tuberculous. Mast cells and basophils often accompany eosinophils, and 5% to 10% may be seen in pleural fluid eosinophilia⁴ (see Table 30-3).

Mesothelial cells in small numbers are normally sloughed into the serous cavities. During inflammatory processes, they proliferate and are shed in greater numbers. They often vary in appearance, manifesting reactive or atypical changes, which can create difficulty during the differential examination (see Figs. 30-12 and 30-13); familiarity with these issues is necessary when examining the slides. Mesothelial cells may appear as single cells, in clusters, or sheets. Clustering can be an artifact caused by cytocentrifugation that may produce a resemblance to malignant cells. Clumps, or loose aggregates of benign mesothelial cells, can be differentiated from malignant cells by comparing the appearance of those in the clump with other more easily distinguished mesothelial cells in the same smear. A uniform, regular arrangement of cells that display **fenestrations**—openings or “windows” between the cytoplasmic membranes of the cells—usually indicates that they are benign. Spheroidal or multilayered cell formations are more likely to be malignant (see Figs. 30-18 and 30-19). The cells are large, with scant to abundant light gray to deep blue cytoplasm, and may present an area of perinuclear pallor; as such, they may resemble large plasma cells. There may also be variably sized cytoplasmic vacuoles. The nucleus occupies one-third to one-half the cell's diameter and may be round to oval in shape with a smooth nuclear membrane. The chromatin distribution is uniform, with a stippled, dark purple effect with the Wright (Wright-Giemsa) stain. There may also be one to three spherical nucleoli. Mesothelial cells that could be classified as “reactive” have slightly irregular nuclei with prominent nucleoli (Fig. 30-27)

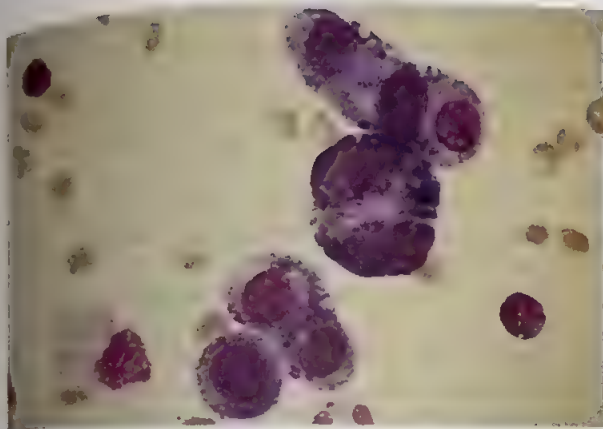


FIGURE 30-27 Reactive mesothelial cells: Pleural fluid; note the prominent nucleoli and multinucleation. Wright stain, $\times 1,000$ magnification. (Courtesy of Judith Brody, M.D., Northwell Health Laboratories, Lake Success, New York.)

ADVANCED CONTENT

Increased concentrations of mesothelial cells are seen in pneumonia, pulmonary infarction, and malignant disorders. A marked increase, especially if the fluid is bloody or eosinophilic, suggests the presence of a pulmonary embolism. However, they are usually markedly reduced in number in cases of TB of the pleura (i.e., tuberculous pleurisy) or if there is massive infection of the pleural cavity with pyogenic (pus-producing) organisms; this may be related to the fibrinous exudate that covers the lining of the cavity, trapping the cells⁹ (see Table 30-3).

Peritoneal Effusions

A peritoneal effusion is an accumulation of fluid in the peritoneal space, clinically termed peritoneal, ascitic, or paracentesis fluid. The causes of peritoneal effusions are the same as those involved in pleural and pericardial effusions: increased capillary pressure or permeability, abnormal colloid osmotic pressure, poor lymphatic drainage, or cardiac abnormalities. Additional causes include abdominal conditions that do not directly involve the peritoneum, such as hepatic cirrhosis, intrahepatic and portal venous obstruction, hypoalbuminemia, renal dysfunction, ovarian disease, pancreatic disease, and parasitic infection. Malignant disease and alcoholic cirrhosis are the most common causes of peritoneal effusion.⁴

Specimen Collection and Processing

The procedure to withdraw peritoneal fluid is known as paracentesis (see Table 30-5). Paracentesis is most commonly performed to rule out bacterial peritonitis and malignancy. Aspiration may be combined with a **lavage**: a flushing of the peritoneal space with normal saline solution. Evidence of blood in the lavage fluid is an indication for immediate surgical exploration (laparotomy) of the abdominal cavity for internal bleeding.

Because the abdominal cavity is large and distendable, more than approximately 500 mL of fluid must usually be

present before the effusion can be detected by radiological or physical examination. Diagnostic abdominal paracentesis, with the removal of 50 to 100 mL of fluid, is essential to determine a differential diagnosis. A portion of this is sent to the laboratory for analysis in the same manner as discussed previously for pleural and pericardial fluids.

Patients in renal failure, who must undergo dialysis to cleanse the blood of waste products, can be treated with a procedure known as **continuous ambulatory peritoneal dialysis (CAPD)**. This procedure utilizes the natural properties of the peritoneal membrane and the infusion of a dialyzing fluid into the cavity to remove the impurities in the bloodstream. This procedure requires laboratory monitoring to measure its effectiveness and carries an inherent risk of recurrent peritonitis. The procedural dialysis fluid (dialysate) can be sent to the laboratory for analysis.

Laboratory Analysis and Clinical Correlations

Qualitative Analysis The hematology laboratory will receive this fluid in tubes containing EDTA as well. If the specimen was not properly collected or handled, and arrives in the laboratory completely clotted, the analysis cannot be performed. As with pleural and pericardial fluids, a partially clotted specimen would not provide an accurate cell count, but morphological examination for the presence of reactive or malignant cells should be performed, with the required comment on the report and prompt physician notification.

The criteria previously described to differentiate transudates and exudates may not fully apply for ascitic fluid as they do for pleural and pericardial fluids. The total protein and LDH ratios may be better indicators in most cases (see Table 30-1). A more reliable method is known as the **serum-ascites albumin gradient (SAAG)**, calculated by subtracting the ascitic fluid albumin concentration from the simultaneously collected serum albumin concentration.¹⁰ The SAAG is significantly greater than 1.1 g/dL in transudates (high-gradient ascites) than in exudates.^{10,11} It can also provide valuable information to assist in the differential diagnosis of ascites (e.g., patients with high-gradient ascites include those with cirrhosis, alcoholic hepatitis, cardiac-related ascites, or massive liver metastasis). Low-gradient ascites (SAAG less than 1.1 g/dL) are associated with peritoneal malignancies and nonmalignant diseases such as TB, pancreatitis, nephrotic syndrome, biliary disease, or connective tissue diseases.^{12,13}

Normal or transudative peritoneal fluid is pale yellow or straw-colored and transparent. The visible appearance of the fluid may be helpful in determining the cause of the effusion. A grossly bloody fluid may be caused by or indicate abdominal blunt trauma, postoperative complications, rupture of the liver or spleen, intestinal infarction, pancreatitis, or malignancies. Effusions resulting from perforation of the gallbladder or intestines, duodenal ulcers, cholecystitis, or acute pancreatitis will appear green due to the presence of bile. Exudative effusions will be cloudy or turbid if they contain elevated protein concentrations, increased leukocytes, and/or microorganisms. Chylous fluid is rare, but it can be caused by the leakage of lymphatic vessels due to trauma, hepatic cirrhosis, TB, lymphoma, or carcinoma.

Quantitative Microscopic Analysis Next, the specimen is examined for its cellular concentration. RBC and TNC counts are performed on the undiluted fluid. Cloudy or turbid fluids may be diluted with saline or buffered cell diluent for manual counting and the cell count multiplied by the appropriate dilution factor. Automated analyzers can provide RBC, TNC, and WBC counts. The total RBC and WBC counts are useful for diagnosis in peritoneal lavage, as they improve the accuracy and specificity of the diagnosis. A positive lavage fluid would be visibly bloody and considered as such when the total RBC count is greater than 100,000/ μ L (or greater than 50,000/ μ L in known penetrating trauma such as stab or gunshot wounds). The total WBC count would be greater than 500/ μ L. However, when indeterminate by eye, a lavage would be considered positive when the total RBC count is 50,000 to 100,000/ μ L (10,000 to 50,000/ μ L in cases of penetrating trauma).⁴ The total WBC count would be 100 to 500/ μ L. A negative lavage fluid would show a total RBC count less than 50,000/ μ L (or less than 1,000/ μ L in penetrating trauma). The total WBC count should be less than 100/ μ L.

Cell counts on nonlavage peritoneal fluids, specifically the total WBC count, are of limited value in differential diagnoses but are useful in distinguishing peritoneal transudates from exudative effusions. A WBC count greater than 300/ μ L is considered to be abnormal. It is extremely necessary to make a correct diagnosis as early as possible in suspected spontaneous bacterial peritonitis (SBP) to reduce the morbidity and mortality associated with it. A WBC count greater than 500/ μ L is useful presumptive evidence in distinguishing between an exudate in bacterial peritonitis and a transudate of cirrhosis. Serial WBC counts and cultures are helpful in assessing the adequacy of treatment in patients with SBP and also in differentiating SBP from nonperforation secondary bacterial peritonitis.⁴

A Wright (Wright-Giemsa)-stained cytocentrifuge preparation is used for the differential examination. The same procedure, as described earlier in the discussion of pleural and pericardial effusions, is followed for the morphological examination. An exudative peritoneal effusion characteristically contains a variable number of neutrophils, lymphocytes, eosinophils, basophils, macrophages, and mesothelial cells.

Morphological Analysis The presence of more than 25% segmented neutrophils is considered abnormal and suggestive of bacterial infection. The absolute neutrophil count may also be helpful; counts greater than 500 neutrophils/ μ L are a sensitive indicator of spontaneous or secondary bacterial peritonitis. In CAPD, peritonitis is defined by a WBC count of greater than 100/ μ L with more than 50% neutrophils or if microorganisms are seen on the cytocentrifuge preparation and/or Gram stain of the dialysate.⁴ Chronic, long-standing effusions may show neutrophils with decreased cytoplasmic granules and nuclear pyknosis and karyorrhexis as in pleural or pericardial effusions.

Lymphocytes transform in response to various stimuli and exhibit a variety of morphological features. Differentiation of benign lymphocytes from malignant lymphoma in effusions may occasionally be difficult. A predominance of lymphocytes can be seen in transudates from patients with congestive heart

failure, cirrhosis, or the nephrotic syndrome; they may also be seen in chylous effusions, tuberculous peritonitis, and other malignant disorders.

Eosinophilia in peritoneal fluid is less commonly seen than in pleural fluid. Eosinophilic ascites is rare; when present, it contains greater than 50% eosinophils and is associated with congestive heart failure, vasculitis, and malignant lymphoma.¹⁴ Patients with continuous ambulatory peritoneal dialysis (CAPD) may exhibit peritoneal eosinophilia, usually greater than 10% or as high as 95%.¹⁴⁻¹⁶ The cause is unknown but may be caused by hypersensitivity to the foreign material or the possibility that air entered the cavity during the connection of the catheter line to the infusion set¹⁶ (see Table 30-3). Small numbers of basophils and mast cells may also be seen in cases of peritoneal eosinophilia.

It is possible to find choroid plexus cells from the CNS in the peritoneal fluid of a patient with a **ventriculoperitoneal shunt**, which is a drainage device inserted into the ventricles of the brain to remove excess CSF in cases of hydrocephalus, neoplastic conditions, or head injury. The catheter is placed under the skin, from the skull to the abdomen, allowing the excess fluid to drain into the peritoneal cavity, where it is reabsorbed. A one-way valve controls the flow of the fluid.

Mononuclear phagocytes are present in variable numbers. These cells, as well as mesothelial cells, may have a variable appearance. As discussed previously under pleural and pericardial fluid analysis, differentiation from malignant cells may be difficult. Atypical mesothelial cells, resembling malignant cells, are especially seen in chronic effusions and in ascitic fluid associated with cirrhosis.¹⁷ LE cells have also been reported. Referral to pathology and cytological examination must be performed if a malignancy is suspected.

Cerebrospinal Fluid (CSF)

The CSF is a selective ultrafiltrate of plasma, under the control of the blood-brain and blood-CSF barriers and differs from serous fluids in that a greater volume is present in the normal state. This fluid is actively secreted by cells in the ventricles and subarachnoid space. Examination of CSF is an extremely important diagnostic procedure, and its acquisition is not without risk to the patient. All specimens must be handled with universal precautions and never discarded until all testing has been completed.

Specimen Collection and Processing

Proper specimen collection is of the highest importance to provide the most clinically accurate information. The physician must perform a complete clinical history and physical examination before the collection procedure. Indications for the analysis of CSF are divided into four major categories: suspicion of meningeal infection, subarachnoid hemorrhage, CNS malignancy, and demyelinating diseases.¹⁸ Imaging techniques such as computerized tomography (CT scan) and magnetic resonance imaging (MRI) can provide clinical information without the need for fluid analysis in cases of intracranial masses. However, laboratory analysis of CSF remains critical to the diagnosis of CNS infections and inflammatory

conditions, especially in neonates or infants younger than 12 months of age with the presence of fever and lethargy.

CSF collection is commonly performed by intervertebral puncture in the lumbar region of the spinal column, between vertebrae L3 and L4, known as a **lumbar puncture (LP)** or "spinal tap" (see Table 30-5). The procedure must be performed aseptically and without trauma. In patients with coagulopathies or infection at the puncture site, LP is contraindicated. If the patient has a normal intracranial pressure, determined at the initiation of the LP, at least 20 mL of fluid can be removed in an adult without complication. Significantly lower volumes may be collected from infants, children, and neonates, or if the intracranial pressure is elevated. Approximately 2 to 4 mL of fluid is collected into each of three to five sequentially numbered, sterile, nonadditive tubes. The tubes must be filled in numerical order. Tube #1 is used for chemical analysis because it typically would be contaminated with peripheral blood and cellular debris from the initiation of the puncture and requires centrifugation before analysis. Tube #2 is used for microbiological analysis as it is less affected by contamination from initiation of the LP. Tube #3 is sent to hematology for the cell count and differential analysis as it is less affected by any bleeding from initiation of the LP. Additional tubes (#4, #5) may be used for further biochemical analyses, serology, cytology, flow cytometry, immunocytochemistry, or molecular genetic analysis. An LP may also be performed for therapeutic purposes, such as to reduce intracranial pressure and/or to administer anesthetics, radiographic contrast media, antifungal, or chemotherapeutic agents directly into the CNS. Whole blood samples are also collected at the time of LP for a complete blood count (CBC) and chemical analysis of the serum.

On receipt in the laboratory, the CSF must be processed immediately (STAT); analysis must occur within 1 hour of collection due to imminent cellular degradation and lysis that will lead to falsely lower WBC and RBC counts. The lower protein content of CSF leads to destabilization of the cell membranes. Any specimen remaining after analysis should be promptly refrigerated in case additional tests are necessary. An exception to this procedure applies only to specimens for microbiological examination; these are not refrigerated, as some microorganisms (e.g., *Neisseria meningitidis*) are destroyed by low temperatures. It is preferable to place the specimen for microbiology evaluation in a 37°C incubator until testing is performed, especially during off-hours when the bacteriology department at smaller institutions may not be staffed.

CRITICAL THINKING QUESTION

30-2 Why must CSF collection follow strict guidelines in the filling of the sterile tubes?

Laboratory Analysis and Clinical Correlations

Qualitative Analysis

The first step in CSF analysis is a gross inspection for the tube number that was received, along with a physical evaluation examining volume, clarity, color, and viscosity of the

fluid. A normal CSF is clear, colorless, sterile, and watery in consistency. Any cloudiness or turbidity can be caused by the presence of more than 200 WBC/ μ L, more than 400 RBC/ μ L, microorganisms, fat globules, or increased protein levels. The presence of more than a normal number of cells, particularly WBC, in CSF is referred to as **pleocytosis**. Radiographic contrast media can give the fluid an oily appearance.

Grossly bloody specimens may result from a traumatic tap, which occurs in approximately 20% of LPs, or the presence of subarachnoid or intracerebral hemorrhage.¹⁹ A common problem in the analysis of CSF is distinguishing between a true CNS hemorrhage versus a traumatic LP. If the bloodiness decreases in each tube in sequential order, it suggests a traumatic tap. If the bloodiness is similar in all tubes, it could indicate a CNS hemorrhage. Physicians may request the cell count on successive tubes for this purpose. However, this method is not always reliable.²⁰

A clotted CSF is an abnormal finding; it suggests an increased fibrinogen and/or protein concentration from peripheral blood contamination, an infectious condition within the CNS, or a markedly increased protein concentration in the fluid.

The presence of any color should be noted. If the specimen is bloody, the color of the supernatant is interpreted after centrifugation. This must be performed as soon as all the cell counts are completed. **Xanthochromia**, a pink, orange, or yellow color of the supernatant, is caused by the breakdown of hemoglobin. This is usually thought to indicate a true CNS hemorrhage and is present in more than 90% of patients within 12 hours of subarachnoid hemorrhage onset.²¹ Lysis of RBCs present in the CSF begins approximately 1 to 2 hours after a subarachnoid hemorrhage. Hemoglobin is converted to bilirubin within 24 hours of RBC lysis, giving the supernatant a yellow tint. If the fluid supernatant is clear and colorless, this could suggest that the LP was traumatic, the CNS hemorrhage occurred less than 2 hours before the LP, or minimal RBC lysis has occurred. Xanthochromia will occur, however, if a bloody fluid from a traumatic LP is not processed within 1 hour of collection. Other causes of xanthochromia include jaundice (serum bilirubin levels of 10 to 15 mg/dL), extremely elevated CSF TP concentrations (greater than 150 mg/dL), and hyperbilirubinemia in premature infants who have an immature blood-CSF barrier.

Quantitative Microscopic Analysis

Next, the specimen is examined microscopically. The numbers of RBCs and TNC in the undiluted fluid are manually counted using a hemacytometer (see Chapter 31). Cloudy or bloody fluids may be diluted with saline or buffered cell diluent, correcting the final count by the dilution factor. As described earlier in the chapter, automated cell counters should not be used for CSF because the allowable background limits of the analyzer diluent may be higher than the normal range for CSF cell counts.

Normal CSF is practically acellular by the manual hemacytometer count (Table 30-6). No RBCs should be present (less than 1/ μ L); RBC counts are of limited value in a differential diagnosis. Erythrocytes can be present due to trauma during LP and are commonly seen in infants. Nucleated cell counts,

TABLE 30-6 Normal Values for Cerebrospinal Fluid

Appearance	Clear, colorless, watery
RBC Count	<1/ μ L
WBC Count	0–5/ μ L (adults) 0–30/ μ L (infants up to 1 year)
WBC Differential	3% neutrophils ~70:30% lymphocytes:monocytes; macrophages; rare ependymal cells

however, are very useful in developing a differential diagnosis because even low counts are suggestive of pathology. Normally, less than 5 mononuclear cells/ μ L can be seen in adults; in neonates and infants up to 1 year of age, up to 30 mononuclear cells/ μ L is possible. These cells commonly consist of lymphocytes, monocytes/macrophages, or CNS tissue cells.

The specimen is processed by cytocentrifugation and Wright (Wright-Giemsa) staining, as previously discussed under serous fluid analysis. A normal CSF can yield 30 to 50 cells per 0.5 mL of a cytocentrifuged fluid sample.¹⁹

A standard 100-cell differential is then performed under high-power oil immersion (50 \times or 100 \times objective). If fewer than 100 cells are present, all the cells in the preparation are counted, with the actual numbers of cells counted and the percentages of each cell type being calculated and reported. The total WBC count can be calculated from the total percentages of the leukocytes seen on differential exam and the total nucleated cell count in the hemacytometer. WBC counts greater than 1,000/ μ L with turbid fluid suggest bacterial or fungal meningitis; very high counts (greater than 50,000 cells/ μ L) are unusual and suggest intraventricular rupture of a brain abscess. The total WBC count cannot be interpreted without the total RBC count. In a traumatic LP, the WBC and RBC counts will reflect the same WBC/RBC ratio as the peripheral blood of the patient. Generally, in a patient with a normal peripheral blood count, one can expect to find approximately one to two WBCs for every 1,000 RBCs in the CSF from a traumatic LP.²² For example, a CSF with a total WBC count of 10 cells/ μ L and an RBC count of 10,000 RBCs/ μ L, the ratio is 1:1,000. There is no significant increase in WBCs in this fluid; however, a traumatic LP is implicated. In a CSF with a total WBC count of 10 cells/ μ L and an RBC count of 100 RBCs/ μ L, the ratio is 1:10, indicating a significant increase and a pathological state.⁴ In a situation in which a patient has an increased or decreased peripheral blood WBC or RBC count, a correction to the count of the CSF must be performed to see if it is significant, using the following formula:^{4,18}

$$\text{WBCs added} = \frac{\text{WBC (in blood)} \times \text{RBC (in CSF)}}{\text{RBC (in blood)}}$$

$$\text{Corrected (true) CSF WBC} = \text{total WBC (in CSF)} - \text{WBCs added}$$

The types of WBCs present is the most important aspect in the interpretation of a CSF and their relationship to the clinical findings.

CRITICAL THINKING QUESTION

30-3 Why must CSF cell counts not be performed via automated body fluid methods?

Morphological Analysis and Microorganisms

On differential analysis, a normal CSF contains mononuclear cells (Fig. 30–28) (approximately 70% lymphocytes and 30% monocytes) and rare ependymal or choroid plexus cells. Other cells that could be seen in a CSF include granulocytes (mature and immature), lymphocytes (mature and/or reactive), mononuclear phagocytes (monocytes, histiocytes, and macrophages), plasma cells, ependymal and choroidal cells, leukemic blasts, and malignant cells. The cells can appear comparable to those in peripheral blood with some variation in details.

Neutrophils Normally, very few segmented neutrophils should be observed in CSF. They traditionally were considered pathological; the observation of more than an occasional neutrophil classically suggested a bacterial infection. However, with the current method of cytocentrifugation that concentrates the fluid and improves cell recovery, the appearance of a small number of neutrophils can be seen in normal CSF. These may be related to peripheral blood contamination from a traumatic LP; their concentration would be proportional to the extent of peripheral blood contamination and the neutrophil percentage of the peripheral blood. There is no general agreement on what constitutes a normal upper limit to the neutrophil count, but neutrophil counts greater than 3% should be regarded as potentially pathological and carefully investigated.¹⁸ Their quantity must always be considered in the context of the patient's clinical condition and with results of other laboratory tests. Neutrophils rapidly disintegrate; if the specimen is not examined promptly, a falsely decreased count can potentially be reported. The cytoplasmic granules are often less prominent than in the peripheral blood.^{23,24} The nucleus may be hyperlobulated with long and narrow filaments, an artifact of cytocentrifugation. Older cells can exhibit pyknosis or karyorrhexis and might be mistaken for NRBCs.

Bacterial infections of the CNS cause neutrophilic pleocytosis and increased total WBC counts. Early bacterial meningitis may



FIGURE 30-28 Normal CSF; two monocytes and one lymphocyte (A). Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

show only a slight increase in neutrophils. Early viral meningitis (first 48 hours) may also present with neutrophilia. Increased numbers of neutrophils can also be seen in inflammatory or non-infectious conditions, such as tissue infarction, foreign body or substance, metastatic tumor/leukemic infiltration, or 3 to 4 days after a CNS hemorrhage. The fluid differential alone cannot differentiate between bacterial and nonbacterial meningitis.

Lymphocytes Normal CSF contains lymphocytes, which are predominantly small, with intermediate and large forms present as well. They appear similar to those in the peripheral blood and undergo transformation in response to antigens. Transformed or reactive lymphocytes are variable in size, with moderately abundant basophilic cytoplasm, moderately coarse chromatin, and one or more nucleoli; some may appear plasmacytoid. They may also occasionally contain azurophilic granules (Fig. 30-29). When marked reactive changes occur, it may be difficult to identify them as lymphocytes. Lymphoblasts, or immature-appearing lymphocytes, have scant cytoplasm, a fine chromatin pattern, and may or may not have prominent nucleoli. An increased number of lymphocytes (lymphocytic pleocytosis; Fig. 30-30) is associated with viral, fungal, tuberculous, syphilitic, and parasitic infections.

Reactive lymphocytosis is seen in viral meningoencephalitis, or after chemotherapy or radiation treatment. The reactive lymphocytes present in viral meningitis are morphologically very similar to those in the peripheral blood during infectious mononucleosis. Noninfectious and degenerative causes of reactive lymphocytosis include MS, Guillain-Barré syndrome, drug therapy, polyneuritis, and sarcoidosis of the meninges. In MS, the reactive lymphocytes are distinctly plasmacytoid in appearance (Fig. 30-31). Their nuclei are eccentric and lymphocytic in appearance, whereas their cytoplasm is plasma cell-like. Blast-like lymphocytes are frequently seen in the CSF of newborn infants and can be mistaken for leukemic cells. The ability to differentiate benign and malignant lymphocytes is important. Benign cells appear as a mixture of variable sizes and a mixture of normal and reactive cells. This contrasts with malignant cells that appear more homogeneous in size and shape.

Plasma cells are normally not present in CSF. In inflammatory conditions associated with lymphocytic reactions,

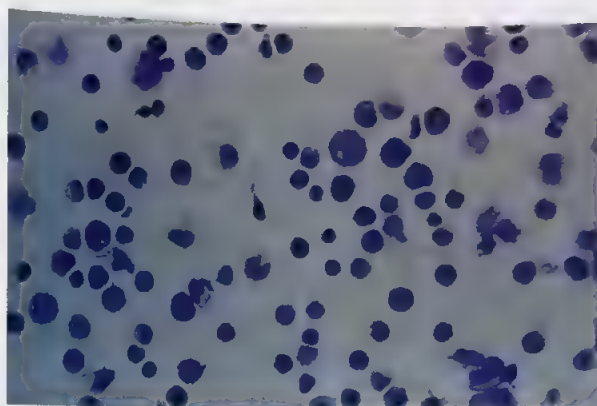


FIGURE 30-30 Lymphocytic pleocytosis, CSF. Wright stain, $\times 400$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

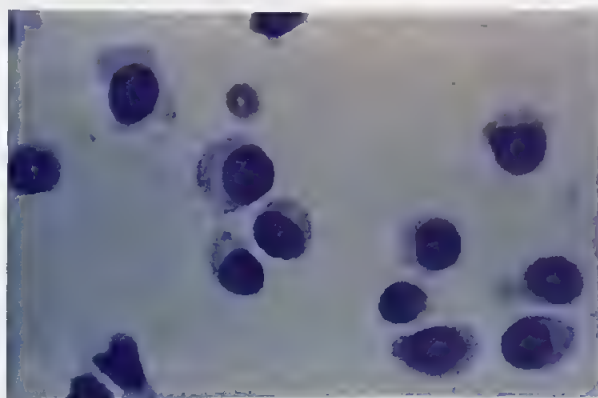


FIGURE 30-31 Reactive (plasmacytoid) lymphocytes in CSF in multiple sclerosis. Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

transitional stages of reactive lymphocytes, plasmacytoid lymphocytes, and plasma cells can be seen. These conditions include acute viral infections, chronic inflammatory states, TB, syphilis, sarcoidosis, Guillain-Barré syndrome, and MS.

Monocytes Monocytes present in CSF appear similar to, and arise from, the peripheral blood. They normally constitute approximately 30% of the cells seen on differential examination, they can be more numerous in infants and small children. Differentiation from lymphocytes can be problematic in some cases, especially in neonates. Cyto centrifugation may cause the monocytes to stick together and appear like choroidal, ependymal cells, or tumor cells.¹⁸ They degenerate more rapidly in vitro, requiring prompt preparation of the slide. They are increased in a variety of disorders, including tuberculous meningitis, fungal meningitis, syphilitic meningoencephalitis, and viral meningoencephalitis. Pure monocytosis is rarely seen in the CSF. More commonly, they present as part of a mixed cell reaction, which includes neutrophils, lymphocytes, and plasma cells.

A definitive sign of CNS hemorrhage is **erythrophagocytosis**, the phagocytosis of erythrocytes by histiocytes or macrophages. It takes approximately 18 hours for histiocytes or macrophages to mobilize and phagocytose RBCs after the hemorrhage, thus appearing as empty vacuoles within the macrophages. More than



FIGURE 30-29 Reactive lymphocytosis in CSF. Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

a single macrophage containing ingested RBCs should be present before considering a CNS hemorrhage as they may occur *in vitro* or if an LP is repeated 8 to 12 hours after an initial traumatic LP. After approximately 4 days, the degraded hemoglobin becomes **hemosiderin**, which is visible as dark brown or black granules (Fig. 30–32). If the hemorrhage is older, **hematoidin crystals**, a product of hemoglobin catabolism, may be seen as bright yellow or red crystals (Fig. 30–33). Iron-containing macrophages, known as **siderophages**, are visible with the Prussian blue stain (stain for iron).

Eosinophils and Basophils Eosinophils are rarely seen in a normal CSF. They may be increased in a variety of infectious and noninfectious disorders. Eosinophilic meningitis is defined when the total WBC count is greater than 10% eosinophils; a parasitic infection should be suspected with this finding.²⁵ Idiopathic eosinophilic meningitis, without any evidence of a pathogen, has also been described.²⁶ Other infectious causes include viral, fungal, or rickettsial infections. Noninfectious causes include malignancy, intrathecal therapy, radiographic contrast media, and systemic drug reactions. Eosinophilia is also associated with malfunctioning ventricular shunts or foreign body reactions (Fig. 30–34). Basophils, as well, are normally not seen

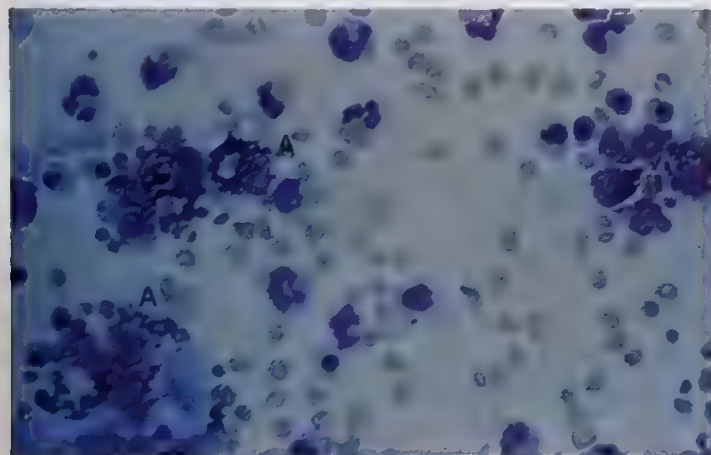


FIGURE 30-32 Siderophages in CSF: hemosiderin pigment in macrophages (A). Wright stain, ×400 magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

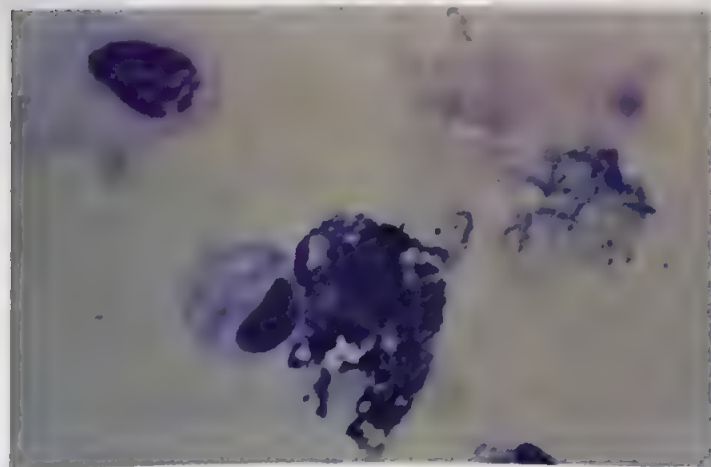


FIGURE 30-33 Siderophage with hematoidin (hematin) pigment. Wright stain, ×1,000 magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

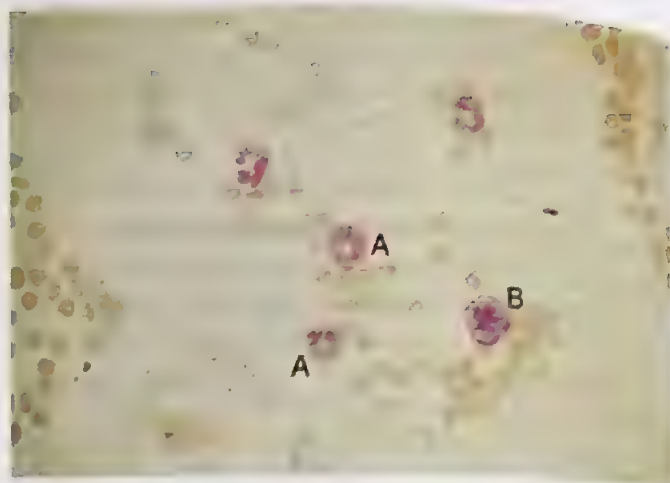


FIGURE 30-34 Eosinophils (A) and reactive lymphocytes (B) in CSF; ventricular shunt. Wright stain, ×1,000 magnification. (Courtesy of Judith Brody, M.D., Northwell Health Laboratories, Lake Success, New York.)

in CSF. There are conditions in which they can be found in small numbers, such as inflammatory diseases, foreign body reactions, parasitic infections, convulsive disorders, and chronic myelogenous leukemia (CML).¹⁸

ADVANCED CONTENT

Ependymal or choroid plexus cells that line the cerebral ventricles and choroid plexus, sometimes referred to as neuroectodermal cells, may be seen in CSF under certain circumstances. They are rarely seen in normal CSF obtained by LP in adults; however, they may occasionally be found in infants.¹⁸ These cells are medium in size and may appear in papillary clusters or sheets, or sometimes individually. The nuclei are the size of a small lymphocyte, round to oval in shape, with delicate, finely granular, and evenly distributed chromatin. Nucleoli are not present (Figs. 30–35 and 30–36). Their uniformity of nuclear size and appearance in sheets or as single cells are helpful features in their identification. Occasionally, the nucleus may be pyknotic and eccentrically placed. The cytoplasm

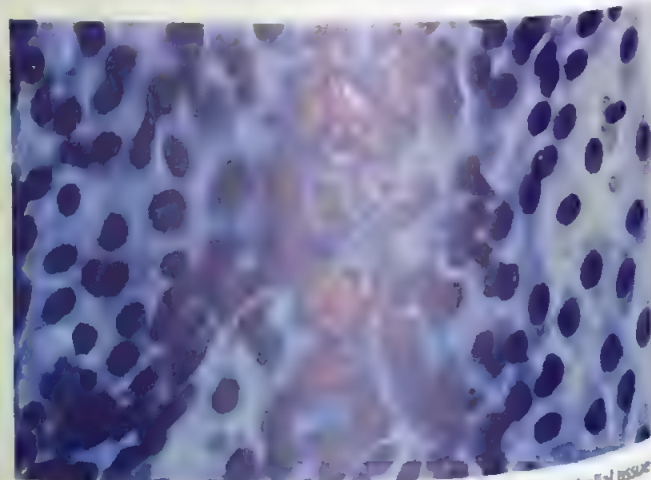


FIGURE 30-35 Ependymal or choroid plexus cells (neuroepithelial tissue) in sheet formation. Wright stain, ×400 magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

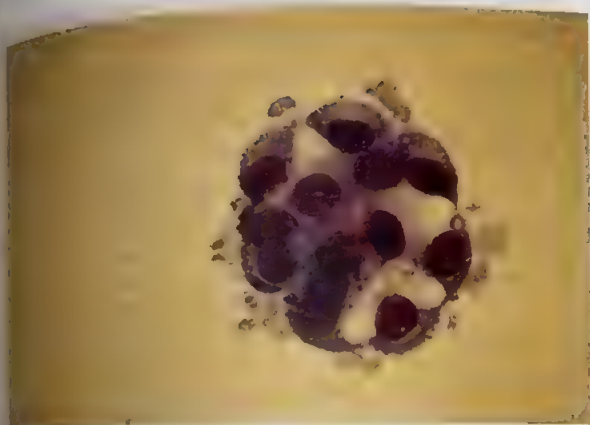


FIGURE 30-36 Papillary cluster of neuroepithelial lining cells. Wright stain, $\times 1,000$ magnification. (Courtesy of Judith Brody, M.D., Northwell Health Laboratories, Lake Success, New York.)

is moderate to abundant, stains a gray-blue with the Wright (Wright-Giemsa) stain, and may contain vacuoles; the cytoplasmic borders may be indefinite, and cilia may occasionally be present.¹⁸ Situations or conditions in which these cells may be seen include CSF obtained by ventricular tap following a traumatic brain injury, brain surgery, ischemic brain infarction, radiological procedures, and in children with hydrocephalus and ventricular shunts.^{18,27} It is important to recognize these cells because they can be mistakenly identified as malignant cells; however, they have no diagnostic significance.

Malignant Cells Malignant cells can be seen in the CSF. Every Wright (Wright-Giemsa) stained CSF slide must be thoroughly examined for the presence of clumps of tumor cells. The presence of any large tissue cells should be considered suspicious for malignancy. Carcinoma cells tend to be less cohesive; they may simulate hematopoietic malignancies. Free tumor cells can originate from primary CNS neoplasms or metastases from tumors of the lung, breast, gastrointestinal tract, or melanoma. Cytology studies are required for identification. Leukemic cells in the CSF are rarely an initial finding, and their appearance usually indicates established disease. Their infiltration of the CNS presents a treatment dilemma. The blood-CSF barrier is almost impermeable to chemotherapeutic agents, allowing the disease to progress uninhibited. Intrathecal chemotherapy must be instituted to kill the malignant cells and any latent cells within the CNS from the CNS disease. Acute lymphoblastic leukemia involves peripheral blood contamination with leukemic cells; if no RBCs are present, even 1% to 2% of blasts present may be indicative of CNS involvement. Malignant lymphomas may involve the CNS, with the presence of lymphoma cells in the CSF (Figs. 30-38, 30-39, and 30-40). These may also be

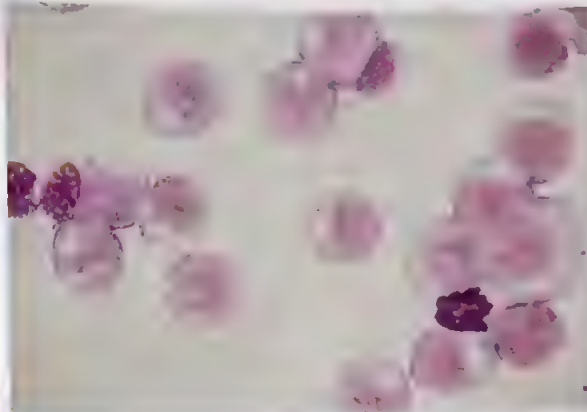


FIGURE 30-37 Acute lymphoblastic leukemia in CSF; note the prominent nucleoli. Wright stain, $\times 1,000$ magnification. (Courtesy of Judith Brody, M.D., Northwell Health Laboratories, Lake Success, New York.)

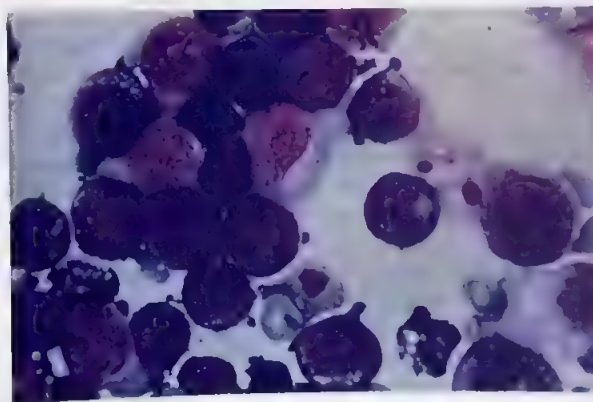


FIGURE 30-38 Burkitt's lymphoma in CSF. Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

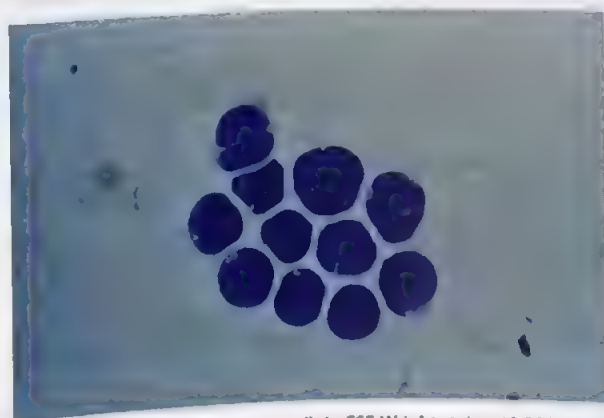


FIGURE 30-39 Cleaved lymphoma cells in CSF. Wright stain, $\times 1,000$ magnification. (From Best, M. Body Fluids Examination. Boston: ASCP Workshop 9294; 1990, with permission.)

difficult to differentiate from leukemic cells. The presence of lymphoma cells in the CSF shows a direct correlation with bone marrow involvement and leukemic conversion of the lymphoma. Any questionable cellular morphology must be reviewed by the pathologist.

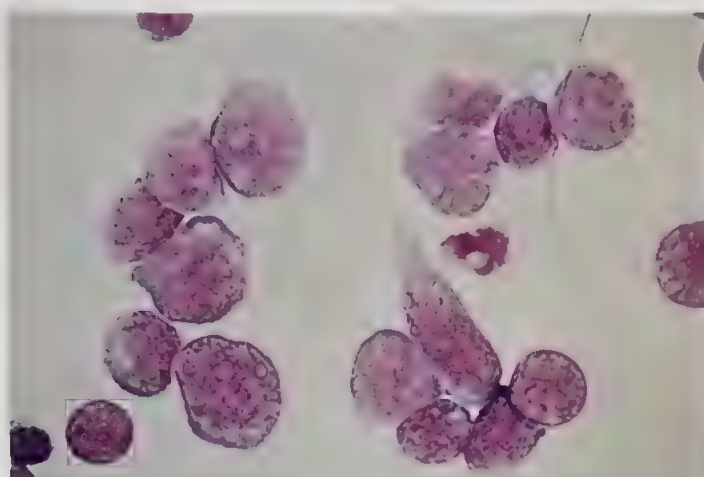


FIGURE 30-40 Lymphoma cells in CSF. Wright stain, $\times 1,000$ magnification. (Courtesy of Judith Brody, M.D., Northwell Health Laboratories, Lake Success, New York.)

Microorganisms Microorganisms may be present in CSF samples; they may be intra- or extracellular and must be distinguished from stain precipitate. A Gram stain, performed by the microbiology department, is helpful in this situation. The most common yeast organism to infect the CNS is *Cryptococcus neoformans*. Cultures must be performed if any organisms are present. Viral cultures can also be performed, although these can take up to 7 days to obtain a definitive result. Some viral studies can now be performed using the polymerase chain reaction (PCR), which can provide results within a day. PCR is used to detect viral DNA in a specimen and is an extremely sensitive method. Viruses frequently implicated in CNS infection are Herpes simplex viruses (HSV), cytomegalovirus (CMV), human immunodeficiency virus (HIV), and enteroviruses.

Synovial Fluid

The analysis of synovial fluid provides important information in the diagnosis of inflammatory and degenerative joint diseases. Age, as well as many inflammatory and pathological joint disorders, can alter the volume and composition of the synovial fluid affecting its function. An increased volume of synovial fluid is considered an effusion and classified as inflammatory or noninflammatory, septic, or hemorrhagic. Inflammatory responses caused by mechanical, chemical, immunological, or bacterial damage change its cellular and chemical constitution. The impaired function of the fluid may play a role in the development of degenerative joint diseases. Normal values for routine synovial fluid analyses are summarized in Table 30-7.

Synovial fluid is most often analyzed when there is suspicion of infectious (septic) arthritis or a crystal-associated disease of the joint (e.g., gout). A delay in diagnosing septic arthritis can lead to serious complications, which include joint destruction and long-term disability. Because there is no laboratory or radiological test that can rule out septic arthritis, this analysis is critical to the proper management of the patient.²⁸ This condition is more common in children than adults, with the highest incidence occurring between 6 months and 3 years

TABLE 30-7 Normal Values for Synovial Fluid

Feature	Description
Appearance	Transparent, colorless to straw-colored
Physical Qualities	Colorless Viscous; does not clot
WBC Count	$<200/\mu\text{L}$
Differential	Neutrophils: $<25\%$; remainder as lymphocytes, monocytes, synovial cells, macrophages
RBC Count	None
Total Protein	1–3 g/dL
Glucose	≤ 10 mg/dL serum–fluid difference
Crystals	None
Uric Acid	Equivalent to normal serum level

of age.^{29,30} Trauma, which could introduce pathogenic organisms into the joint space, is a possible cause; however, an upper respiratory infection, otitis media, or other infected site can predispose the formation of septic arthritis, spread by the bloodstream. Other diseases may not be able to be diagnosed by synovial fluid examination alone; clinical history, physical examination, and radiological studies may also be necessary.

Specimen Collection and Processing

The analysis of synovial fluid begins with the aspiration of fluid in a procedure known as **arthrocentesis**; this must be performed only by trained medical professionals by aseptic technique. Arthrocentesis may also be used therapeutically, to alleviate increased intra-articular pressure, thereby removing inflammatory products and limiting potential joint damage (see Table 30-5).

Testing needs to be initiated within 1 hour of collection unless the specimen is refrigerated. This timing is important due to cellular degradation and chemical changes that occur with time. If a sufficient volume of fluid is submitted, a routine analysis should include an examination of a wet preparation for crystals, a cell count and differential analysis, a Gram stain and culture, and chemical analysis for protein and glucose concentrations.

Laboratory Analysis and Clinical Correlations

Qualitative Analysis

Once the specimen is received by the laboratory, it is examined grossly for volume, color, clarity, viscosity, and clot formation. Normal synovial fluid is transparent, colorless, viscous, and does not clot. Viscous fluids can represent normal or noninflammatory states and generally decreases with inflammation due to lytic enzymes that depolymerize the hyaluronic acid. This reduces the lubricating ability of the fluid, promoting damage to the joint. If the physician requires the assessment of fluid viscosity, it should be performed at the time of aspiration (not in the laboratory).

The color of synovial fluid changes with the disease process and is dependent on the quantity of albumin, bilirubin,

and other debris present. A normal fluid may be colorless to straw-colored. Noninflammatory, inflammatory, and infectious fluids can have a yellow color due to hemoglobin breakdown of RBCs that enter the synovial cavity during inflammation or from chromogenic products of bacteria within the joint cavity. A traumatic aspirate will show streaks of blood in the fluid. **Hemarthrosis**—hemorrhage within a joint—produces a homogeneously bloody fluid. This can be caused by disease (e.g., hemophilia) or a traumatic injury. Centrifugation is necessary to determine the origin of the fluid. A xanthochromic supernatant (similar to CSF) indicates that breakdown of RBCs has occurred; a bloody or dark red-brown supernatant is suggestive of hemarthrosis rather than a traumatic aspirate or injury.

Fluid clarity also changes with the disease process. Turbidity increases with inflammation caused by leukocytes, crystals, or cartilage debris. Septic arthritis produces a purulent fluid caused by the presence of bacteria and pus. The presence of crystals can create a cloudy, turbid, fatty, or milky appearance. Large quantities of degenerated synovial lining cells can create the appearance of pus, commonly seen in patients with RA.

Spontaneous clotting of fluid is observed in inflammatory conditions. Fibrinogen and other clotting proteins are acute phase reactants, which can be present in synovial fluid. A grossly bloody fluid caused by a traumatic aspiration or traumatic injury will produce spontaneous clotting due to plasma fibrinogen; a hemorrhagic effusion would not clot.

Quantitative Microscopic Analysis

The microscopic examination of synovial fluid is crucial to determining the presence of crystals and leukocytes. Septic, inflammatory, and noninflammatory effusions are classified by the quantity and types of leukocytes present; the crystal-induced joint diseases are characterized by the presence of crystals. A fresh synovial fluid must be screened by performing a wet preparation; a drop of well-mixed, anticoagulated fluid is placed on an exceptionally clean microscope slide with a clean coverslip and the edges sealed with clear nail polish or petroleum jelly to prevent drying. It is then examined by routine microscopy for cells, crystals, and other particulates.

The hematology laboratory will receive the fluid in tubes containing EDTA, which should prevent clotting, although very viscous fluids may form small clots if they are bloody and/or not well mixed immediately following collection. If the specimen is partially clotted, the cell count would be inaccurate, but an attempt should be made at performing the morphological examination for the leukocyte differential, with the required comment on the report and prompt physician notification. If the fluid is completely clotted, no analysis can be performed.

A TNC and RBC count can be performed manually with a hemacytometer. Debris within the specimen may clog the flow of automated analyzers, and crystals or fat globules may be erroneously counted as cellular material. Viscous fluids are difficult to pipette; they can be pretreated with either 0.05% hyaluronidase in phosphate buffer (i.e., one drop per milliliter of fluid) or with a pinch of lyophilized hyaluronidase to liquefy

the sample. Fluids should be counted undiluted and allowed to settle in the counting chamber for at least 30 minutes. If the appearance of the fluid suggests that dilution is necessary (i.e., bloody and/or turbid), only isotonic (0.85% or 0.9%) or phosphate buffered saline must be used as the diluent. Other diluents may contain acetic acid that will precipitate the hyaluronic acid, trapping cells and falsely lowering the cell count. If the fluid is bloody, it may be necessary to lyse the RBCs in a small aliquot of the sample with 0.3% saline or 0.1 N hydrochloric acid to count the nucleated cells more accurately.²² RBCs are lysed so they do not overdilute the nucleated cells (i.e., the greater the dilution factor, the more error that may be introduced). A separate count must then be performed for the RBCs alone. A normal synovial fluid does not contain red blood cells; however, an RBC count less than 2,000/ μ L is considered normal. The concentration of RBCs is rarely of clinical significance unless there is a need to determine whether a traumatic tap has occurred.

The WBC count, derived from the differential analysis and total nucleated cell count, is necessary to classify the type of effusion present, but by itself is not diagnostic. A normal fluid will have less than 200 WBC/ μ L. Noninflammatory effusions can contain 200 to 3,000 WBC/ μ L, and inflammatory effusions can contain 3,000 to 75,000 WBC/ μ L. Infectious disorders can contain 50,000 to 200,000 WBC/ μ L, and the crystal-induced disorders are associated with WBC counts of 500 to 200,000/ μ L.⁴ There is obviously a considerable amount of overlap to the leukocyte counts among disorders. The counts need to be considered in the context of the clinical presentation and, most importantly, the differential cell count and morphology, which is critical for differential diagnosis in synovial fluid analysis.

Morphological Analysis

Morphological examination is performed using Wright (Wright-Giemsa)-stained cytocentrifuge preparations. The cells that are normally present include lymphocytes, macrophages, and synovial cells. Neutrophils are also present but do not exceed 25% of the total cells.

Abnormal synovial fluids can contain neutrophils, lymphocytes (normal and reactive), plasma cells, eosinophils, histiocytes, macrophages, synovial cells, and LE cells. In acute inflammatory conditions, synovial fluid can contain many neutrophils and a moderate number of synovial cells; bacteria may also be present. In chronic inflammation, synovial fluid contains lymphocytes, plasma cells, and histiocytes. In RA, synovial fluid may contain many plasma cells and moderate numbers of lymphocytes, histiocytes, synovial cells, cartilage cells, and rarely multinucleated giant cells.

Certain diagnoses can be suspected when comparing the total WBC count with the percentage of segmented neutrophils present. If the differential count reveals 90% or more segmented neutrophils, then an infectious agent is most likely present. A Gram stain and culture must be obtained, microorganisms can be seen in synovial fluid if present in sufficient numbers, and they should be considered to be intracellular organisms. However, if bacteria are absent on Gram stain, this would not exclude the possibility that there is sepsis in

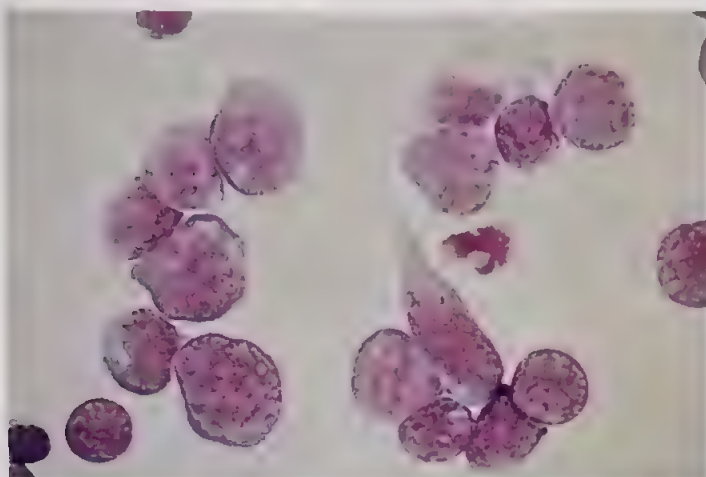


FIGURE 30-40 Lymphoma cells in CSF. Wright stain, $\times 1,000$ magnification. (Courtesy of Judith Brody, M.D., Northwell Health Laboratories, Lake Success, New York.)

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Certain diagnoses can be suspected when comparing the total WBC count with the percentage of segmented neutrophils present. If the differential count reveals 90% or more segmented neutrophils, then an infectious agent is most likely present. A Gram stain and culture must be obtained, microorganisms can be seen in synovial fluid if present in sufficient numbers, and they should be considered to be intracellular organisms. However, if bacteria are absent on Gram stain, this would not exclude the possibility that there is sepsis in

the synovial cavity. Bacterial organisms are more common; pathogenic yeasts are seen only rarely. If fluids have WBC counts in the range of 2,000 to 200,000/ μL , with greater than 50% neutrophils, a possible diagnosis of RA, SLE, or Reiter's syndrome should be considered. **Reiter's syndrome** is a reactive arthritis caused by intestinal bacteria that also affect the skin, eyes, and muscles. The presence of LE cells is suggestive of SLE but not diagnostic as they can also be seen in RA.

Eosinophilia of the synovial fluid, defined as greater than 2% of the total WBC count, can be associated with RA, rheumatic fever, metastatic carcinoma, parasitic infestation of the joint, Lyme disease, radiological procedures, and radiation therapy.⁴

ADVANCED CONTENT

Other types of cells that can be observed in inflammatory synovial fluids are known as **RA cells** and **Reiter's cells**. RA cells are associated with RA but are not specific for a diagnosis of RA. These cells may also be called "ragocytes" or "inclusion body cells." These are neutrophils that may contain from 1 to 20 granules in the cytoplasm and are known to contain immune complexes such as IgG, IgM, complement, and rheumatoid factor. Reiter's cells—also nonspecific and not diagnostic for Reiter's syndrome—are vacuolated macrophages with intracytoplasmic inclusions or debris of ingested neutrophils, which may appear as unrecognizable blue material with the Wright (Wright-Giemsa) stain.

Synovial lining cells are mononuclear cells with morphology resembling that of the mesothelial cells in serous fluids. Their nuclei may have small, regular nucleoli. They may become proliferative in a reactive setting similar to mesothelial cells. Reactive synovial cells may be multinucleated and may occur in clusters. Synovial lining cells may also be difficult to differentiate from macrophages and histiocytes. Their presence does not have any specific diagnostic significance.

Malignant cells can be seen in synovial fluid, although this is extremely rare; they are usually derived from metastatic disease.

Crystal Analysis and Clinical Correlations

Every synovial fluid sent to the laboratory for a cell count must be examined for crystals, especially if infection is not a consideration. This aspect of fluid analysis has the greatest effect on diagnosis and therapy. Extra cytocentrifuge slides should be prepared and left unstained to determine whether crystals can be seen before staining. If few crystals are present, phase-contrast or polarized light microscopy may be necessary to see them initially by wet preparation or cytocentrifugation. Synovial fluids should be thoroughly examined for crystals, for at least 15 minutes, before they are reported as negative. Artifacts such as cell clumps or fibrin strands may obscure or trap the few crystals that may be present.

Polarized light must be used for the detection and confirmation of **birefringence**, which is the ability of a particular material to refract light (Fig. 30-41). This can be determined

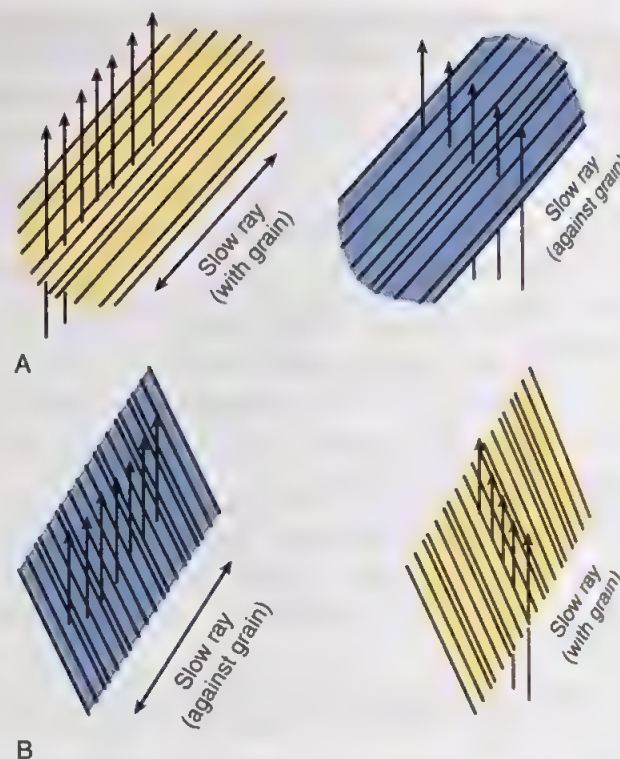


FIGURE 30-41 Negative and positive birefringence in MSU and CPPD crystals. **A** – MSU crystal with grain running parallel to the long axis producing a negative (yellow) birefringence. **B** – CPPD crystal with grain running perpendicular to the long axis producing a positive (blue) birefringence.

using a polarized light microscope that contains a rotating and a fixed filter. The rotating filter, known as a **polarizer**, is situated below the slide stage in the light path. A fixed filter, known as the **analyzer**, is located above and between the objectives and the oculars. Both filters will allow only light of one direction, or **polarity**, to exit. When the polarizer is rotated 90 degrees with respect to the analyzer, it creates a block, or **maximum extinction** of the polarized light, yielding a dark field. If the specimen contains birefringent material, the direction of the light is refracted, allowing this light to pass through the analyzer and is seen as a bright particle or crystal against the dark field. Not all materials possess birefringent characteristics, but this attribute assists in identification of the particles present in the synovial fluid. Another filter, known as a **red compensator**, changes the velocity of the transmitted light, allowing birefringent crystals to display different colors depending on their position in relation to the axis of the compensator. This is used to further identify and confirm the existence of certain types of crystals.

In addition, if unknown crystals are seen, **Alizarin Red S** stain can be used to stain any calcium that may be present in the crystals. A drop of the stain is added to a drop of the synovial fluid, placed on a slide, and cover slipped. Crystals containing calcium appear orange with light microscopy or bright red under polarized light.

Laboratories that perform crystal analysis and identification must use appropriate quality control materials to align the polarized microscopes properly and for comparative purposes in identification.

The causes of crystal deposition are not well understood, but certain predisposing factors may exist such as increasing age, the presence of joint damage, and familial inheritance. Others include metabolic disorders, osteoarthritis (OA), and SLE. The most common types of crystals that may be present in synovial fluid include monosodium urate (MSU) and calcium pyrophosphate dihydrate (CPPD). Less common crystals and artifacts also occur, which may be confused with clinically significant synovial fluid crystals or interfere in their detection.

Monosodium Urate (MSU) Crystals

The presence of MSU crystals is pathognomonic for gout, causing a condition known as gouty arthritis. These crystals are seen in the majority of patients during acute attacks. Between attacks, they may be seen in approximately 75% of patients.⁴ They may also be present, occasionally, in cases of inflammation. MSU crystals are typically long, thin, and needle-like with pointed ends. They may be seen singly or in aggregates; rarely, they may appear as spherules that are composed of clusters of many individual needle-shaped crystals.³¹ Some Wright-staining methods can cause MSU crystals to dissolve, which is why the wet preparation must always be examined first. These crystals may be found within neutrophils or macrophages during attacks of acute gouty arthritis.^{4,31,32} It is important to report their appearance as intra- and/or extracellular; phagocytosis of crystals suggests that they are responsible for the acute arthritis.³² MSU crystals are strongly birefringent and appear white in polarized light; however, they lose their birefringence with compensated polarization. These characteristics are sufficient to identify them as MSU crystals. If these crystals are parallel to the axis of the compensator, they will appear yellow (negative birefringence); if perpendicular they will appear blue (Fig. 30-42). As previously mentioned, an increased concentration of uric acid in the serum and/or synovial fluid, even without the presence of MSU crystals, is diagnostic of gout.

Calcium Pyrophosphate Dihydrate (CPPD) Crystals

CPPD crystals are characteristically present in a group of disorders known as CPPD deposition disease (CPDD).³³ It is also known as pseudogout or chondrocalcinosis. These crystals are a common cause of arthritis, most frequently seen in the elderly, and in patients with degenerative arthritis.



FIGURE 30-42 MSU crystals under compensated polarized light. The yellow crystals are parallel to axis. (Photo courtesy of Stony Brook University.)

Patients with hereditary forms of CPDD, as well as metabolic disorders, may also possess CPPD crystals. The symptoms of this condition may imitate gout or RA. Occasionally, a synovial fluid may contain both MSU and CPPD crystals; the presence of one does not exclude the other. This may be an indication of septic arthritis, especially if the WBC count is extremely elevated. These crystals may also be seen intra- and/or extracellularly. They can be more difficult to identify, typically appearing as short rectangular shapes, but they may also assume multiple three-dimensional forms such as rods and rhomboids. Needle-shaped forms are also possible, causing misidentification as MSU crystals. They are more easily seen with light microscopy; they are only weakly birefringent and therefore may be difficult to see with polarized light. With compensated polarization, these crystals have the opposite appearance of MSU. They appear blue if parallel to the axis (positive birefringence) and yellow if perpendicular, thereby differentiating them from MSU crystals (Fig. 30-43). The use of Alizarin Red S staining will indicate the presence of calcium.

Other Crystals

Crystals of calcium oxalate may be found in the synovial fluid of patients with primary oxalosis, a rare inborn error of metabolism, but is more commonly seen in patients with chronic renal failure who undergo hemodialysis. The deposition of these crystals causes arthropathy. The crystals appear as small, bipyramidal or pleomorphic shapes, with a wide variation in birefringence and stain positively with Alizarin Red S.

Cholesterol crystals can be present in chronically inflamed joints and are not diagnostic of any particular disorder. They may also be found in chylous effusions that contain a high concentration of lipids. Although rarely observed, they are most often seen in cases of patients with long histories of RA,³⁴ OA, and ankylosing spondylitis.³⁵ Crystal formation is thought to involve the cholesterol from the cell membranes of degenerating cells associated with impaired drainage of the joint.⁴ They have characteristically large, rectangular notched-plate shapes that are easier to see by regular light microscopy, although strongly birefringent with polarization.

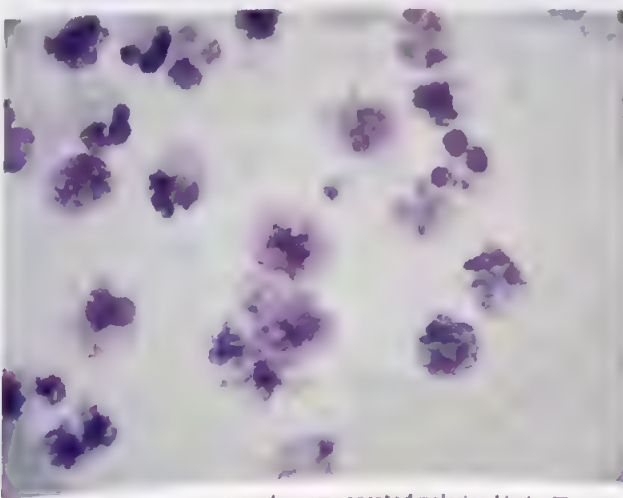


FIGURE 30-43 CPPD crystals under compensated polarized light. The blue crystals are parallel to axis. (Photo courtesy of Kathy Finnegan.)

Lipids can exist in crystalline and noncrystalline forms and are typically found in both chylous and chronic effusions.^{4,22} Noncrystalline lipids occur as fat globules that are round and nonbirefringent. Lipid crystals are round when seen by light microscopy. With polarized light, the crystalline forms appear as strongly birefringent Maltese crosses. Under compensated polarization, they appear as red and blue crystals. Lipid crystals can be mistaken for MSU spherules by an inexperienced analyst. However, observation under high-power magnification ($\times 100$ objective) would reveal they are not composed of numerous individual crystals.

Artifacts

Numerous particulates can appear in synovial fluid during microscopic analysis that are clinically insignificant and/or not originally of synovial origin. These are known as **artifacts** and must be differentiated from clinically significant synovial particulates. Most artifacts such as dust, glass fragments, lint, and fibers can be avoided with the

use of clean microscope slides and coverslips. Cartilage fragments, collagen fibrils, or fat globules may be present due to a disease process or as a result of the arthrocentesis; metallic fragments may arise from articular prostheses. As previously mentioned, therapeutic intra-articular steroid injections can cause the formation of crystalline structures. Wright (Wright-Giemsa)-stained slides may contain stain precipitates. Artifactual crystals typically have variable appearances by regular light and polarized light microscopy. They do not have definite crystal morphology. Clinically significant crystals have regular outlines with smooth, parallel edges. Polarized light microscopy can create confusion in differentiating artifacts from pathological crystals, most commonly when starch granules enter the specimen from surgical gloves. They appear as variably to strongly birefringent Maltese crosses, similar to lipid crystals. In addition, they can be mistaken for MSU spherules. However, starch granules have irregular outlines and a central depression visible by light microscopy.

SUMMARY CHART

- Fluids from the thoracic and abdominal cavities are referred to as **serous fluids**; they are ultrafiltrates of plasma.
- The pleural, pericardial, and peritoneal cavities are lined by a membrane composed of a single layer of mesothelial cells.
- Normal pleural, pericardial, and peritoneal cavities do not contain appreciable amounts of fluid.
- The subarachnoid space contains cerebrospinal fluid (CSF), which is a selective ultrafiltrate of plasma.
- Joints (articulations) are enclosed by a joint capsule; the synovium or synovial membrane is composed of synovial cells.
- Important terms: **Thoracentesis** = removal of pleural fluid; **Pericardiocentesis** = removal of pericardial fluid; **Paracentesis** = removal of peritoneal fluid; **Lumbar puncture** = collection of CSF; **Arthrocentesis** = removal of synovial fluid; **Effusion** = an abnormal collection of fluid in a body cavity; **Transudates** = effusions caused by a systemic disease state; **Exudates** = effusions caused by a primary pathological state within the compartment; **Chylous effusions** = exudates resulting from leaking or blocked lymphatic vessels.
- Transudates result from increased capillary hydrostatic pressure or decreased plasma oncotic pressure.
- Exudates result from increased capillary permeability and/or decreased lymphatic reabsorption.
- All body fluid specimens must be processed immediately upon receipt in the laboratory and all specimens are evaluated for the appropriate collection container and visually inspected for volume, color, clarity, and/or the presence of fibrin clot. Serous fluid is collected into EDTA for hematological analysis.
- Laboratory tests performed by the hematology laboratory include total cell count and morphological and differential analysis. Synovial fluids are additionally evaluated for crystals.
- Body fluid slides for morphological analysis are prepared by cytocentrifugation. This method markedly improves the quality of the cell morphology obtained but may create artifacts. These slides are used for the WBC differential count and for detection of any reactive or malignant cells.
- CSF is collected into each of three to five sequentially numbered, sterile, nonadditive tubes. The tubes must be filled in numerical order.
- Xanthochromia = a pink, orange, or yellow color of the CSF supernatant caused by the breakdown of hemoglobin. It is usually thought to indicate a true CNS hemorrhage. Xanthochromia will occur, however, if a bloody fluid from a traumatic LP is not processed within 1 hour of collection.
- Normal synovial fluid is transparent, colorless, viscous, and does not clot.
- The analysis of synovial fluid provides important information in the diagnosis of inflammatory and degenerative joint diseases.
- The most common types of crystals that may be present in synovial fluid include monosodium urate (MSU) and calcium pyrophosphate dihydrate (CPPD).

CASE STUDY 30-1

A 50-year-old man with no prior medical history sought medical attention for flu-like symptoms. The patient has a current history significant for a persistent cough and fever of 2 weeks' duration. Antibiotic therapy was prescribed by the physician and the patient was sent home with the directive to increase fluid intake and bedrest. After 2 days, severe left-sided chest pain and shortness of breath developed. The patient was brought to the emergency room. A radiological exam revealed a collection of fluid in the left chest cavity. The fluid was withdrawn for analysis, and a simultaneous peripheral blood specimen was collected.

The laboratory values obtained on the chest fluid and peripheral blood are indicated:

Serum total protein:	6.0 g/dL
Serum LDH:	50 U/L
Fluid appearance:	Thick, turbid, yellow
Fluid total protein:	4.5 g/dL
Total protein ratio:	0.75
Fluid LDH:	40 U/L
LDH ratio:	0.8
Fluid WBC:	20,000/ μ L
Differential:	Neutrophils = 90%
	Macrophages = 10%
	Many degenerating cells present

QUESTIONS

1. What is the name of the fluid collection in the chest cavity?
2. What is the name of the procedure to withdraw this fluid?

3. Is the fluid specimen identified as a transudate or an exudate?
4. Is the fluid specimen chylous?
5. What further tests should be performed on the fluid specimen?
6. Are these findings representative of an acute or chronic reactive process?

ANSWERS

1. A fluid collection in the chest cavity is known as a pleural effusion.
2. The procedure to withdraw a pleural effusion is known as thoracentesis.
3. The pleural effusion collected is considered to be an exudate because the WBC count is greater than 1,000/ μ L, the total protein is greater than 3 g/dL, the total protein ratio is greater than 0.5, and the LDH ratio is greater than 0.6.
4. This specimen is not chylous, as the predominant cell type in the differential examination are neutrophils, whereas chylous effusions predominantly contain lymphocytes. It could be considered pseudochylous by its appearance, but laboratory studies for triglycerides are not available.
5. Further tests to be performed should include a Gram stain and culture.
6. These findings are representative of an acute reactive process, which present with greater than 50% neutrophils on differential examination.

CASE STUDY 30-2

A 2-year-old male child was brought to the emergency room to determine why he suddenly was refusing to walk and complaining of pain in his right leg. There was no history of trauma. He had been seen by his pediatrician earlier in the day due to the presence of fever and an upper respiratory infection; he was diagnosed with otitis media, prescribed an antibiotic, and sent home.

Physical exam in the emergency room revealed pain and tenderness of the right ankle. There was also some edema and erythema. X-ray exams of the lower extremities did not reveal any fractures or displacements. Laboratory tests were ordered on his peripheral blood, and a fluid aspirate was taken from the ankle. The results were as follows:

Peripheral Blood:

WBC:	26,000/ μ L
Differential:	Neutrophils = 75%
	Bands = 2%
	Lymphocytes = 19%
	Macrophages = 4%
RBC parameters:	Normal
Erythrocyte sedimentation rate:	Elevated
Plasma glucose:	96 mg/dL

Fluid Analysis:

Color:	Yellow
Clarity:	Purulent
WBC:	22,000/ μ L
Differential:	Neutrophils = 95%
	Macrophages = 5%
RBC:	375,000/ μ L
Fluid glucose:	39 mg/dL

Continued

CASE STUDY 30-2—cont'd**QUESTIONS**

1. What type of procedure was performed to obtain the joint fluid, and what terminology applies to the specimen?
2. What abnormal results are present, if any, and what could they indicate?
3. What additional tests should be performed to aid in diagnosis?

ANSWERS

1. The procedure to obtain fluid from a joint space is known as arthrocentesis; the fluid collected is known as synovial fluid.
2. The child's peripheral blood WBC count and erythrocyte sedimentation rate are elevated but consistent with the presence of an acute upper respiratory infection or other inflammatory process. However, the fluid analysis reveals abnormalities in the clarity, cell counts and differential, and the glucose level. These parameters

indicate the presence of an active inflammatory process; a normal synovial fluid would not contain RBCs, there would be less than 200 WBC/ μ L, and neutrophils would not exceed 25% of the WBCs. The presence of neutrophilia (greater than 80%) would be highly suggestive of septic arthritis, regardless of the WBC count. Results can overlap disease categories, and it is possible for more than one type of disease process to be present simultaneously. Clinical presentation and the microscopic findings, most importantly the differential cell count and morphology, will determine what process is involved and what course of action should be taken.

3. Additional testing must include examination of a Gram stain and culture of the fluid to identify the presence of bacterial organisms. If positive, this would point to a diagnosis of septic arthritis.

CASE STUDY 30-3

A 50-year-old man with a history of alcohol abuse was seen in the emergency room for symptoms of nausea, vomiting, and abdominal pain. On physical exam, the abdomen appeared distended. A computerized tomographic (CT) scan of the abdomen revealed a collection of fluid. About 1,200 mL of fluid was drained and portions were sent to the laboratory for analysis. Peripheral blood was collected simultaneously. The laboratory findings were as follows:

Serum Values:

Glucose	99 g/dL
Total protein	7 g/dL
Albumin	4.0 g/dL
LDH	60 U/L

Fluid Analysis:

	Clear, pale yellow
Glucose	100 mg/dL
Total protein	1.9 g/dL
TP ratio	0.3
LDH	25 U/L
LDH ratio	0.4
Albumin	2.4 g/dL
SAAG	1.6
RBC	500/ μ L
WBC	60/ μ L
Differential:	Neutrophils = 10%
	Lymphocytes = 80%
	Monocytes/Macrophages = 10%
	Atypical mesothelial cells seen

QUESTIONS

1. What type of procedure was performed to drain and collect the fluid, what body cavity was involved, and what terminology applies to the specimen?
2. Is the fluid a transudate or an exudate?
3. What process(es) could cause the production of this type of fluid?
4. What is the SAAG and what is its significance?
5. What does the presence of atypical mesothelial cells indicate?

ANSWERS

1. Paracentesis is the procedure to drain and collect fluid from the peritoneal cavity. This collection of fluid is known as a peritoneal effusion or ascites. The collected fluid may be referred to as peritoneal fluid, paracentesis fluid, or ascitic fluid.
2. This fluid is a transudate.
3. Transudative ascitic fluids can be caused by increased capillary pressure or permeability, abnormal colloid osmotic pressure, poor lymphatic drainage, or cardiac abnormalities. Additional causes include abdominal conditions that do not directly involve the peritoneum, such as hepatic cirrhosis, intrahepatic and portal venous obstruction, hypoalbuminemia, renal function, ovarian disease, pancreatic disease, or parasitic infection. Alcoholic cirrhosis is one cause of peritoneal effusion, which appears to be the case in this patient. Malignant disease can also produce an effusion.

CASE STUDY 30-3—cont'd

4. The SAAG, or serum-ascites albumin gradient, is a value calculated by subtracting the ascitic fluid albumin concentration from the simultaneously collected serum albumin concentration. This value is useful in differentiating whether the fluid is transudative or exudative in nature; transudative ascitic fluids have an SAAG significantly greater than 1.1 g/dL, whereas exudative fluids are found to be less than 1.1 g/dL. In addition, patients with high-gradient ascites include those with cirrhosis, alcoholic hepatitis, cardiac-related ascites, or massive liver metastasis. Low-gradient ascites are associated with peritoneal malignancies and nonmalignant diseases such as TB, pancreatitis, nephrotic syndrome, biliary disease, or connective tissue diseases.
5. The presence of atypical mesothelial cells are especially seen in chronic effusions and in ascitic fluid associated with cirrhosis.

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REVIEW QUESTIONS

1. A specimen is sent to the laboratory labelled as "synovial fluid." What procedure was used to obtain the specimen?
 - a. Thoracentesis
 - b. Paracentesis
 - c. Arthrocentesis
 - d. Lumbar puncture
2. The presence of MSU crystals is pathognomonic for which disorder?
 - a. Pseudogout
 - b. Osteoarthritis
 - c. Rheumatoid arthritis
 - d. Gout
3. Artifacts from cytocentrifugation can include
 - a. Removal of nucleoli
 - b. Vacuolization
 - c. Removal of granules
 - d. Decreased concentration of cells
4. Finding a macrophage with hemosiderin in a CSF sample indicates
 - a. Traumatic tap
 - b. Viral infection
 - c. Bacterial infection
 - d. Hemorrhage
5. Which is another term for "ascites"?
 - a. Pleural fluid
 - b. Thoracentesis fluid
 - c. Peritoneal fluid
 - d. Pericardial fluid
6. Which of the following is the cell type that forms the lining of the pleural, pericardial, and peritoneal cavities?
 - a. Epithelial
 - b. Endothelial
 - c. Mesothelial
 - d. Ependymal
7. Which of the following best characterizes a transudate?
 - a. Total protein greater than 3.0 g/dL; WBC less than 1,000/ μ L
 - b. Total protein less than 3.0 g/dL; WBC less than 1,000/ μ L
 - c. Total protein less than 3.0 g/dL; WBC greater than 1,000/ μ L
 - d. Total protein greater than 3.0 g/dL; WBC greater than 1,000/ μ L
8. A turbid peritoneal fluid is collected from a patient with suspected peritonitis and a cell count is performed. A 1:100 dilution is prepared, and 6 cells are counted in each of the 4 WBC corner squares of the hemacytometer chamber. What is the final total nucleated cell count?
 - a. 37,500/ μ L
 - b. 1,500/ μ L
 - c. 150/ μ L
 - d. 6,000/ μ L

Continued

REVIEW QUESTIONS—cont'd

9. Which of the following is a disadvantage of cytocentrifugation in the preparation of body fluid slides?
 - a. Cell differentiation is determined by Wright stain
 - b. Cell differentiation is done on a concentrated preparation
 - c. Peripheralization, distortion, and segmentation of nuclei
 - d. Normal, reactive, and malignant cells can be identified
10. Which of the following is contained in normal cerebrospinal fluid?
 - a. Lymphocytes and ependymal cells
 - b. Ependymal and choroidal cells
 - c. Mesothelial and ependymal cells
 - d. Erythrocytes and leukocytes
11. What is the purpose for laboratory assessment of synovial fluid?
 - a. Prevention of cardiovascular disease
 - b. Identification of stroke patients
 - c. Diagnosis of nervous system disorders
 - d. Link to degenerative disorders
12. Birefringence is
 - a. Ability to utilize dark light
 - b. Ability to refract light
 - c. Ability to fluoresce
 - d. Ability to reveal other components

See answers at the back of this book.

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Hematology Methods

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CHAPTER OUTLINE

Analytical Phases of Testing

Specimen Collection

- Patient Identification
- Safety
- Verification of Laboratory Requisitions
- Method 31-1. Venipuncture
- Method 31-2. Capillary Blood Collection
- Labeling the Blood Specimen

Specimen Accessioning

Manual Cell Counts

- Method 31-3. Red Blood Cell Counts
- Method 31-4. White Blood Cell Counts
- Method 31-5. Platelet Counts

Evaluation of Peripheral Blood Smear

- Method 31-6. Slide Preparation and Wright Stain
- Alternate Staining Options
- Method 31-7. The White Blood Cell Differential

Methods Used in Detection and Monitoring of Anemia

- Method 31-8. Hemoglobin Determination
- Method 31-9. Microhematocrit Determination
- Method 31-10. Red Blood Cell Indices
- Method 31-11. Reticulocyte Counts
- Method 31-11A. Reticulocyte Counts Using the Miller Disc

Standard Methods for Specific Anemias

- Method 31-12. Sickledex™
- Method 31-13. Helena SPIFE® Alkaline Hemoglobin Electrophoresis
- Method 31-14. Helena SPIFE® Acid Hemoglobin Electrophoresis
- Method 31-15. Hemoglobin A₂ Determination
- Method 31-16. Isoelectric Focusing
- Method 31-17. Hemoglobin F Acid Stain

Method 31-18. Screening Test for Glucose-6-Phosphate Dehydrogenase Deficiency

- Method 31-19. Staining for Heinz Bodies
- Method 31-20. Screening Method for Detection of Red Cell Pyruvate Kinase

Nonspecific Tests of Inflammation

- Method 31-21. Westergren Erythrocyte Sedimentation Rate
- Method 31-22. Alifax® Test 1 Automated Erythrocyte Sedimentation Rate Analyzer

Case Study 31-1

- Summary Chart
- Review Questions
- References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 31-1** Correlate erroneous analytical outcomes with potential pre analytical or analytical causes.
- 31-2** Identify the acceptable specimen type and anticoagulant for hematology analysis.
- 31-3** Describe the limitations associated with the use of capillary blood for hematology analysis.
- 31-4** Assess the use of a hemacytometer counting chamber to perform manual erythrocyte, leukocyte, and platelet counts, including the areas used for counting each and formula used to calculate results.
- 31-5** Evaluate the appropriate staining technique for evaluation of peripheral blood smear, identifying which cellular components are made visible by which stains.
- 31-6** Identify the suggested reference ranges for percent and absolute leukocyte counts.
- 31-7** Evaluate patient hematology data for the possible state of anemia.
- 31-8** Correlate hematology methods with the specific anemic states that they are intended to detect.
- 31-9** Describe the potential indications of abnormal erythrocyte sedimentation rate results.

Standard methods in hematology are a heterogeneous collection of tests that are utilized in the hematology laboratory. Some of these methods may be performed on a routine basis, while others may serve as a backup method when automation is not available or as a specialized test to confirm a particular type of anemia. The results of hematology tests aid the clinician in diagnosing a plethora of hematologic disorders.

Analytical Phases of Testing

Laboratory testing can be broken down into three phases: preanalytical, analytical, and postanalytical. Close adherence to lab protocol in all phases is crucial to produce accurate results. Preanalytical lab errors contribute up to 70% of all lab errors, while postanalytical errors range from 15% to 25%. Analytical errors have been documented at 8% to 15%.

The preanalytical phase of clinical testing is the most frequent cause of laboratory errors that involves collection, transport, and handling of laboratory samples. Sample processing involving proper patient identification and labeling also falls under the preanalytical umbrella.

A common preanalytical complication involves the inadvertent introduction of IV fluid into the hematologic sample. The patient appears anemic with a hematological profile that often involves reticulocytopenia, combined with thrombocytopenia and leukocytopenia. This anomaly is most frequently associated with a dilutional effect associated with improperly collected specimens from or above an IV catheter. The introduction of IV fluid dilutes the sample, causing decreases in cellular numbers.

Hemolysis represents another common preanalytical problem in a hematologic specimen. A small-bore needle used in phlebotomy, vigorous shaking, freezing, or forcing blood through a needle into a collection tube are the most common causes of specimen hemolysis. Hemolysis typically interferes with hemoglobin analysis in automated analyzers, thus serving as a helpful indicator when reviewing RBC indices. Results in a hemolyzed sample tend to exhibit reduced red blood cell count, hematocrit, and mean corpuscular volume (MCV). An increased red blood cell distribution width (RDW), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet count are often present.

Lipemia also erroneously affects sample analysis in hematology. Lipemia is most often correlated with inadequate time of blood collection after eating or too soon after parenteral administration of lipid emulsions to the patient. Lipemic plasma contains high levels of triglycerides and chylomicrons, resulting in falsely increased hemoglobin and MCHC results. A CBC with an MCHC greater than 36 g/dL should be evaluated for lipemia before releasing hematology results.

The analytical phase involves the accuracy, precision, sensitivity, and specificity of a test performed. Daily quality control and patient controls are used to verify instrument accuracy and precision throughout the day.

The postanalytical phase encompasses data transmission, release, and interpretation. Validation studies performed throughout the year establish accurate data transmission when comparing analyzer printouts to the laboratory electronic results. Computer systems are programmed to automatically compare previous and current results (delta check) for significant changes in CBC values. Significant changes observed in a delta check can be reassessed for error, sample integrity, analysis, and sample labeling, or may be explained through clinical changes in a patient.

Specimen Collection

Blood collection for hematologic studies can be performed via venipuncture (blood collected from a vein) or by capillary puncture (blood collected from the heel or finger). The anticoagulant used most often in routine hematological methods, excluding coagulation analysis, is ethylenediaminetetraacetic acid (EDTA). There are many preanalytical steps to follow in the process of blood collection, such as patient identification, order verification of laboratory orders, and biohazard safety, to name a few. These steps, as well as venipuncture and capillary puncture methodology, are discussed here.

Patient Identification

Key identifying patient information varies between hospitalized patients and those being treated in outpatient settings.

Hospitalized Patients

Hospitalized patients wear a wristband with the minimal following information:

1. Julian date
2. Patient's first and last name, middle initial
3. Medical record number
4. Date of birth

This basic information may be expanded depending on health institutional policy. All information should be cross-checked with the laboratory requisition form or laboratory orders for each patient. If the patient is not wearing a wristband, a nurse should be notified to verify patient identification.

Outpatients

For outpatients who have blood collected, the phlebotomist should ask the patient to recite their full name and date of birth. The phlebotomist must check this information with the laboratory requisition form or physician's record of laboratory orders.

Safety

Universal precautions should be in practice at all times when collecting blood specimens.² This means that all specimens should be handled as if they were infected with an infectious agent (e.g., human immunodeficiency virus). Such safety precautions include wearing a new pair of gloves for each patient, washing hands between patients, and disposing of needles and contaminated supplies in a puncture-resistant biohazard container (sharps).

Verification of Laboratory Orders

With each request for blood collection and laboratory analysis, there must be a laboratory requisition that is either written or contained in the laboratory information system (LIS). Information contained on the requisition is outlined in Box 31-1. The phlebotomist must verify that the patient information on the requisition form or specimen label is correct before blood is collected. This verification ensures usage of appropriate anticoagulants for the tests being performed, the order of draw, as well as the volume of blood required.

CRITICAL THINKING QUESTION

31-1 Why would hemolysis of a specimen affect the RBC indices results?

See answers to all Critical Thinking Questions at the back of this book.

Method 31-1. Venipuncture

The most common anticoagulant used in the hematology laboratory is EDTA, and the most common technique for obtaining the specimen is venipuncture using evacuated tubes, which allow for collection of multiple tubes.

METHOD 31-1 Venipuncture	
Principle	After aseptic cleansing of the antecubital fossa, a vacutainer needle is inserted into the patient's vein. An evacuated tube is placed into the vacutainer holder breaching the rubber sheath needle that facilitates flow of blood.
Specimen	EDTA-anticoagulated blood
Procedure	See the required equipment and steps for this procedure at www.fadavis.com

Method 31-2. Capillary Blood Collection

Capillary blood collection serves as an alternative to venipuncture and is most appropriate when microsamples are needed, such as during point-of-care testing and when lesser amounts are required for traditional analysis. Additionally, capillary blood collection is preferred in infants and children less than 2 years old. In adults, capillary blood collection is the preferred collection method for patients with burns, when preserving veins for intravenous treatment such as chemotherapy, and when veins are inaccessible or fragile.

METHOD 31-2 Capillary Blood Collection	
Principle	Using a standard skin puncture device, an aseptic area is punctured approximately 2.0 mm deep and a proximal area of skin is massaged to facilitate adequate blood flow.
Specimen	Capillary blood
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Limitations	Capillary blood may contain interstitial and intracellular fluids that could contribute to inaccurate test results. Failure to disregard the first drop of blood introduces contaminants such as interstitial fluids and disinfectants (alcohol) into the sample. Difficulty in collection or increased pressure to increase blood flow can result in hemolysis.

Labeling the Blood Specimen

A properly labeled specimen is essential to patient care. The consequence of a patient being treated based on another patient's results can be detrimental and sometimes fatal.³ Many hospitals and laboratories have implemented preprinted patient labels that contain the information in Box 31-1. An example of a patient label is depicted in Figure 31-1. There are many varieties of barcode printers.

The tubes in which blood is collected should be labeled after the blood is collected and not before; this reduces

BOX 31-1 Information Present on Laboratory Requisition

- Patient's full name
- Age of patient
- Hospital number
- Date/time of collection
- Physician's name
- Accession or specimen number
- Location where order was placed
- Ordered tests

MR-000-57-9435

ST/ST

SMITH, JONATHAN

DOB: 02/03/XX 24 yrs F 17SEPXX



15U-064C12589

10.00 mLlavender

HE

CPD

SPECIMEN

FIGURE 31-1 Example of a specimen label with bar code.

mislabeling errors. Samples collected must be labeled at the bedside by the collecting phlebotomist. If preprinted labels are not available, the patient's first and last name, medical record number, and date of birth must be written indelibly on the tube of blood. Furthermore, the date and time of collection along with the phlebotomist's initials must be included.

Specimen Accessioning

Once the specimen has been collected, it is accessioned and delivered to the hematology or appropriate department of the laboratory to be analyzed for testing. In specimen accessioning, the information on the printed label is entered into the LIS, which can be done manually or with the aid of a barcode scanner. The patient identifiers as well as the time the specimen was collected and received in the laboratory are permanently entered into the LIS as well as the initials of the phlebotomist.

Manual Cell Counts

Manual cell counts are performed on a hemocytometer counting chamber (Fig. 31-2), which is constructed so that the distance between the bottom of the coverslip and the surface of the counting chamber is 0.1 mm (Fig. 31-3). The surface of the chamber contains two square-ruled areas separated by an H-shaped moat. These two squares are identical, allowing the technologist to duplicate cell counts. Each has a total of 9 mm² (3 mm on each side).⁴ These squares are divided into nine primary squares, each with an area of 1 mm² (1 mm on each side). The four corner primary squares are used when counting leukocytes. These four corner primary squares are further divided into 16 smaller secondary squares, each with an area of 0.04 mm². The four corner and center secondary squares of the center primary square are used to count erythrocytes. All 25 secondary squares of the center primary square are used to count platelets, and each of the 25 squares is further divided into 16 smaller tertiary squares (see Fig. 31-2). Traditionally, glass hemocytometers have been used for

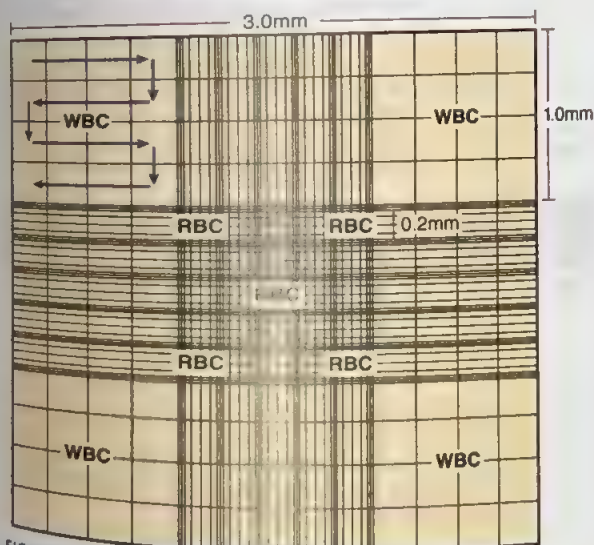


FIGURE 31-2 Spencer Bright-Line double counting system with improved Neubauer ruling. This represents an enlarged view of one of the two ruled squares of the hemocytometer. The four corner primary squares are used for counting white blood cells. The arrows in the upper left corner square represent the suggested counting pathway of cells. Five secondary squares (labeled RBC) of the center primary square are used for counting red blood cells. In platelet enumeration, all 25 squares of the center primary square are counted. (From Wedding, ME, Toenjes, SA. Medical Laboratory Procedures. Philadelphia: FA Davis; 1998, p 277, with permission.)

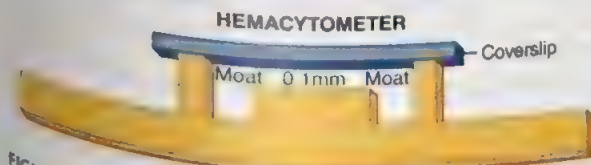


FIGURE 31-3 Hemocytometer, side view.

manual blood counts. Plastic disposable hemocytometers are also available with some offering options for automated enumeration. The use of the plastic hemocytometer has grown in the past few years due to not only being disposable but also the uniformity and ease of using the product.

The boundary lines of the central primary square are either double or triple. When the boundary line is double, all the cells within the square and those touching the innermost line are counted. If the boundary line is triple, all of the cells within the squares and those touching the middle line inward are counted.

Hemocytometers and coverslips should meet the specifications of the National Bureau of Standards and are so marked by the manufacturer. A specific, standardized coverslip must be used that has been ground to fit the specifications of the hemocytometer, ensuring a uniform depth and, therefore, a constant volume. An ordinary coverslip must not be used. Manual cell counts are most often performed in cases of thrombocytopenia.

Method 31-3. Red Blood Cell Counts

Manual RBC counts are rarely performed on whole blood due to innate inaccuracy of counting such a large number of cells. They may, however, still be performed as part of the analysis of certain body fluids.

METHOD 31-3 Red Blood Cell Counts

Principle	EDTA-anticoagulated blood or capillary blood is diluted with sterile 0.9% sodium chloride. The diluted sample is loaded onto a hemocytometer for red blood cell enumeration.	
Specimen	EDTA-anticoagulated blood	
Procedure	See the required equipment and steps for this procedure at www.fadavis.com	
Interpretation	Decreased RBCs are associated with anemia, whereas increased RBCs can indicate polycythemia or dehydration.	
Limitations	The hemocytometer must be properly filled to avoid erroneous results. The rule of counting must be followed to prevent falsely increased results.	
Formulas	$\frac{\# \text{ of cells} \times \text{Dilution factor}}{(\text{area} \times \text{depth})} = \text{Manual cell count}$	
Reporting Results	RBCs may be expressed as RBCs/mm ³ or RBCs $\times 10^{12}/L$. To convert mm ³ to 10 ¹² /L, multiply by a factor of 10 ⁶ .	
Reference Ranges	Newborn	4.4–5.8 $\times 10^{12}/L$
	Infant/child	3.8–5.5 $\times 10^{12}/L$
	Adult men:	4.7–6.1 $\times 10^{12}/L$
	Adult women:	4.2–5.4 $\times 10^{12}/L$

Method 31-4. White Blood Cell Counts

The need for manual white blood counts is most often necessary in body fluid analysis that requires specific enumeration

of the cells present in the sample. Automated counters exhibit linearity levels as low as $0.1 \times 10^3/\mu\text{L}$ white blood cells. Clinically, a white blood cell count under $0.5 \times 10^3/\mu\text{L}$ or lower is considered critical and a specific number is not necessary. Thus, manual white blood cell counts are rarely used for peripheral blood analysis.

METHOD 31-4 White Blood Cell Counts			
Principle	Manual white blood cell counts in peripheral blood require either free-flowing capillary or well-mixed anticoagulated venous blood. A measured volume of a blood sample is added to a specific volume of diluent that lyses the erythrocytes but preserves leukocytes. The diluted blood is added to the hemocytometer chamber. Cells are allowed to settle for 5 minutes before leukocytes are counted.		
Specimen	EDTA-anticoagulated blood or free-flowing capillary blood		
Procedure	See the required equipment and steps for this procedure at www.fadavis.com		
Interpretation	Increased WBCs are most often associated with inflammation and infections.		
Limitations	The hemocytometer must be properly filled to avoid erroneous results caused by underfilling or overfilling. A markedly high leukocyte count may make accurate counting difficult, and a secondary dilution should be made.		
Formulas	$\frac{\# \text{ of cells} \times \text{Dilution factor}}{(\text{area} \times \text{depth})} = \text{Manual cell count}$		
Reporting Results	White blood cell count is reported in thousands per μL or $10^9/\text{L}$		
Reference Ranges	Newborn	9,000–30,000/ μL	$9.0\text{--}30.0 \times 10^9/\text{L}$
	1 week	5,000–21,000 μL	$5.0\text{--}21.0 \times 10^9/\text{L}$
	1 month	5,000–19,500/ μL	$5.0\text{--}19.5 \times 10^9/\text{L}$
	6–12 months	6,000–17,500 μL	$6.0\text{--}17.5 \times 10^9/\text{L}$
	2 years of age	6,200–17,000/ μL	$6.2\text{--}17.0 \times 10^9/\text{L}$
	Child/adult	4,800–10,800/ μL	$4.8\text{--}10.8 \times 10^9/\text{L}$

Method 31-5. Platelet Counts

Manual platelet counts are less often necessary since newer automated analyzers exhibit linear limits down to 1,000 platelets/ μL . Manual platelet counts may be necessary in the presence of large platelets or when automated analyzers cannot discern platelets in a sample.

METHOD 31-5 Platelet Counts	
Principle	Free-flowing capillary or well-mixed anti-coagulated venous blood is added to a 1% ammonium oxalate diluent. The diluent lyses the RBCs but preserves the platelets, white blood cells, and nucleated red blood cells. The diluted blood is added to a hemocytometer counting chamber and allowed to settle for 5 minutes before platelets are counted.
Specimen	Free-flowing capillary or well-mixed EDTA-anticoagulated blood
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	Decreased platelets are associated with bleeding disorders, whereas increased platelets are associated with increased risk of thrombosis.
Limitations	A delay in mixing blood sample (anticoagulated sample) may result in platelet clumping and subsequent erroneous results. The hemocytometer must be properly filled; underfilling or overfilling may lead to erroneous results. In the event of platelet satellitosis (neutrophils ringed with adhesive platelets), obtain correct platelet counts by collecting a fresh specimen with sodium citrate as the anticoagulant.
Formulas	$\frac{\# \text{ of cells} \times \text{Dilution factor}}{(\text{area} \times \text{depth})} = \text{Manual cell count}$
Reporting Results	Results are expressed as number of platelets per μL or mm^3
Reference Ranges	150,000–450,000/ μL or $150\text{--}450 \times 10^9/\text{L}$

CRITICAL THINKING QUESTION

31-2 What do you think is a major contributing factor to the use of disposable hemacytometer counting chambers over the traditional glass ones?

Evaluation of Peripheral Blood Smear

A well-prepared peripheral blood smear provides valuable information concerning the patient's hematologic status and is a vital component of the CBC (complete blood count) when slide preparation is warranted. This section describes slide preparation and the Wright stain, as well as determining the white blood cell differential.

Method 31-6. Slide Preparation and Wright Stain

The wedge blood smear is the most common smear preparation in the hematology laboratory and Wright stain, a Romanowsky stain, is the most common dye. A well-prepared blood smear provides valuable information concerning the patient's hematologic status and is a vital component of the CBC (complete blood count) in the event a slide preparation is warranted.

METHOD 31-6 Slide Preparation and Wright Stain**Principle**

Blood from an EDTA-anticoagulated specimen or capillary blood is applied to a standard glass slide using the slide-to-slide method. The blood smear is then stained using Wright stain. Blood smears are fixed using methanolic fixative solution to stabilize cellular components. Eosin Y is an anionic dye that stains cytoplasm pink to yellowish-red and methylene blue, which stains the nucleus and RNA blue.⁵

Specimen

EDTA-anticoagulated blood

Procedure

See the required equipment and steps for this procedure at www.fadavis.com

Interpretation

Characteristic staining patterns are described in Table 31-1.

Limitations

Only precleaned slides should be used for staining blood smears. It may be difficult to differentiate cells and access cell numbers in a slide that is poorly made or stained. Improper drying can also cause artifactual RBC morphology, such as rouleaux and burr cells. Stain should be free of precipitates to ensure they are not confused with inclusions, platelets, etc.

TABLE 31-1 Romanowsky Staining Pattern of Hematologic Cells

Cellular Constituent	Color
Nuclei	Purple
Myeloblast cytoplasm	Blue
Promyelocyte cytoplasm	Blue
Myelocyte cytoplasm	Pink
Metamyelocyte cytoplasm	Pink
Neutrophil cytoplasm	Pink
Lymphocyte cytoplasm	Blue
Monocyte cytoplasm	Gray-blue
Erythroblast cytoplasm	Dark-blue
Erythrocyte	Pink
Basophilic granules	Purple-black
Neutrophilic granules	Pink-purple
Platelet granules	Pink-purple
Azurophilic granules	Red or purple
Dohle bodies	Blue
Howell-Jolly bodies	Purple
Cabot rings	Pink-purple

Alternate Staining Options

The Hematek® 3000 Slide Stainer (Fig. 31-4) is a popular continuous feed platen-style stainer. It has limited programmability but produces consistently stained smears. The Hematek® 3000 has positions for 25 slides on a metal plate, and slides are stained at a rate of one slide per minute. This instrument has excellent reproducibility and is nearly maintenance free. The Wright's stain is contained in a "stain-pak" from a variety of manufacturers and sits within the instrument connected by stylets and tubing. The Wright stain is triggered to pump and deliver a precise amount of solution to the slide via peristaltic pumps. Once stained, the slide is rinsed and dried on the platen and eventually drops into a separate compartment.

Dried smears can also be stained using an automated dip-style stainer such as a Millipore Sigma™ Midas™ III Automated Stainer (Fig. 31-5). This type of stainer processes smears in batches. Stains are contained within reservoirs, and baskets containing slides are dipped into each reagent specified by the defined program. These stainers



FIGURE 31-4 The Siemens Hematek® 3000 automated platen slide stainer. (Courtesy of Siemens Medical Solutions USA, Inc., Malvern, PA.)



FIGURE 31-5 Millipore Sigma MIDAS III Automated Dip-style Slide Stainer. (Photo Courtesy of Millipore Sigma Company, Burlington, MA.)

can also be programmed to stain bone marrow slides and body fluid smears. Stain baths are changed daily or as needed.

Additional options employ centrifugal force or utilize sprayers to deliver reagents to smears. In centrifugal stainers, slides are positioned within a spinning slide tray and stain is applied at programmed intervals. Aero spraying instruments deliver the staining reagents to smears on a rotating carousel. Both methods are rapid and slide precipitate is minimal.

Automated slide makers/stainers may also be an integral part of a hematology analyzer. These methods produce slides on samples exhibiting lab-specified (abnormal) results, and smear preparation (i.e., wedge angle) is adjusted in accordance

to hematology results. Staining may proceed after slide preparation, if available. In instruments without stainers, prepared slides can be stained as described earlier. Automation can effectively reduce hands-on time, supplies, and turn-around time.

Method 31-7. The White Blood Cell Differential

A total white blood count is generally informative, but the differentiation of the white cells contributes to the ability to, for example, distinguish bacterial and viral infections from allergies and malignant processes. The cells that are present in a peripheral blood cell differential represent physiological responses as well as bone marrow activity.

METHOD 31-7 White Blood Cell Differential

Principle	One hundred white blood cells are counted and classified on a Wright-stained peripheral blood smear using $1,000\times (100\times \text{objective})$ oil immersion magnification.		
Specimen	EDTA-anticoagulated blood		
Procedure	See the required equipment and steps for this procedure at www.fadavis.com		
Interpretation	Neutrophilia with a possible left-shift can indicate inflammation often due to a bacterial infection. Neutropenia can be seen due to medications, in the presence of toxins and in bone marrow replacement. Lymphocytosis, on the other hand, can be caused by viral infections, autoimmune disorders, and lymphoproliferative disease. Since monocytes are phagocytes, they can be seen increased in chronic infections and in a variety of disorders where increased cell death occurs. Eosinophilia can arise in response to an allergy, immunological reaction, or parasitic infection. Leukocyte morphology can be very indicative of a specific disorder whereas the presence of very immature cells in the peripheral blood are often associated with a bone marrow abnormality that will need further investigation.		
Limitations	Improper slide preparation and/or staining can contribute to erroneous identification of cells.		
Formulas	WBC Correction for nRBCs = $\frac{\text{Uncorrected WBC} \times 100}{100 + \text{nRBC}}$		
Reporting Results	Differentials are usually reported in percent cells and absolute cells. Absolute values may be expressed as RBCs/mm ³ or RBCs $\times 10^{12}/\text{L}$. To convert mm ³ to $10^{12}/\text{L}$, multiply by a factor of 10^6 . Absolute counts remain the more sensitive and specific indicator of blood cell population distributions.		
Reference Ranges	Relative counts		
	Lymphocytes	20%–44%	
	Monocytes	2%–9%	
	Neutrophils	50%–70%	
	Bands	2%–6%	
	Eosinophils	0%–4%	
	Basophils	0%–2%	
	Absolute counts		
	Lymphocytes	$1.2\text{--}3.4 \times 10^3/\mu\text{L}$	$1.2\text{--}3.4 \times 10^9/\text{L}$
	Monocytes	$0.11\text{--}0.59 \times 10^3/\mu\text{L}$	$0.11\text{--}0.59 \times 10^9/\text{L}$
	Neutrophils	$1.4\text{--}6.5 \times 10^3/\mu\text{L}$	$1.4\text{--}6.5 \times 10^9/\text{L}$
	Bands	$0\text{--}0.7 \times 10^3/\mu\text{L}$	$0\text{--}0.7 \times 10^9/\text{L}$
	Eosinophils	$0\text{--}0.5 \times 10^3/\mu\text{L}$	$0\text{--}0.5 \times 10^9/\text{L}$
	Basophils	$0\text{--}0.2 \times 10^3/\mu\text{L}$	$0\text{--}0.2 \times 10^9/\text{L}$

CRITICAL THINKING QUESTION

31-3 Is it possible to have a normal relative count for a specific leukocyte, but an abnormal absolute count?

Methods Used in Detection and Monitoring of Anemia

Several methods are used to detect and monitor anemia, including:

- Hemoglobin determination
- Microhematocrit determination
- Red blood cell indices
- Reticulocyte counts
- Reticulocyte counts using the Miller disc

Method 31-8. Hemoglobin Determination

The utilization of the manual hemoglobin determination is rare due to automation. The methodologies of the manual methods have been incorporated into many automated platforms.

METHOD 31-8 Hemoglobin Determination

Principle	Hemoglobin is oxidized to methemoglobin by the reagent potassium ferricyanide or Drabkin's reagent. Methemoglobin is converted to cyanmethemoglobin in the presence of potassium cyanide. The absorbance of cyanmethemoglobin is measured at 540 nm. ⁶ The cyanomethemoglobin method is the internationally accepted method used to calibrate hematology analyzers. ⁷								
Specimen	EDTA-anticoagulated blood								
Procedure	See the required equipment and steps for this procedure at www.fadavis.com								
Interpretation	Slightly lower hemoglobin levels may be normal in some individuals and in pregnant or menstruating women. Low hemoglobin associated with disease states may be due to low production, immunological destruction, or increased or chronic blood loss.								
Limitations	Drabkin's reagent should be kept away from direct sunlight. Adding Drabkin's reagent to samples containing hemoglobin C or S will yield a turbid suspension and will fail to lyse the red cells. To circumvent this, add 5 mL of water to the turbid mixture and incubate at room temperature for 5 minutes. Read off of the spectrophotometer and double the result.								
Reporting Results	Results for hemoglobin are reported in units of g/dL.								
Reference Ranges	<table> <tr> <td>Newborn:</td><td>17–23 g/dL</td></tr> <tr> <td>Infant child:</td><td>9–14 g/dL</td></tr> <tr> <td>Adult men:</td><td>14–18 g/dL</td></tr> <tr> <td>Adult women:</td><td>12–16 g/dL</td></tr> </table>	Newborn:	17–23 g/dL	Infant child:	9–14 g/dL	Adult men:	14–18 g/dL	Adult women:	12–16 g/dL
Newborn:	17–23 g/dL								
Infant child:	9–14 g/dL								
Adult men:	14–18 g/dL								
Adult women:	12–16 g/dL								

Method 31-9. Microhematocrit Determination

The microhematocrit has largely been replaced by automation, in which most analyzers calculate the hematocrit results using generated data. The microhematocrit test is useful in the diagnosis of anemia and polycythemia and is also suitable to monitor treatment response.⁸

METHOD 31-9 Microhematocrit Determination

Principle	EDTA-anticoagulated whole blood or capillary blood is centrifuged, and the total packed red cell volume is expressed as a percentage of the whole blood volume.								
Specimen	EDTA-anticoagulated blood or capillary blood								
Procedure	See the required equipment and steps for this procedure at www.fadavis.com								
Interpretation	A low hematocrit may indicate a decreased number of healthy red blood cells whereas a higher hematocrit may be seen in dehydration, hemoconcentration, or polycythemia.								
Limitations	<p>If capillary blood is used, the first drop should be wiped away in capillary blood collection to avoid contamination with interstitial fluid, which will dilute out the sample.</p> <p>If venous blood from a collection tube is used, ensure proper mixing is performed before filling the capillary tubes for accurate assessment of the red cell population.</p> <p>The buffy coat should not be included in the packed red cell volume reading.</p> <p>Dehydration will cause an increased hematocrit value.</p> <p>If the patient is on chloramphenicol or penicillin, a decreased hematocrit level can be found.⁹</p>								
Reporting Results	The hematocrit is reported as a percentage of red cells to total blood volume.								
Reference Ranges	<table> <tr> <td>Newborn:</td><td>53%–65%</td></tr> <tr> <td>Infant child:</td><td>30%–43%</td></tr> <tr> <td>Adult men:</td><td>42%–52%</td></tr> <tr> <td>Adult women:</td><td>37%–47%</td></tr> </table>	Newborn:	53%–65%	Infant child:	30%–43%	Adult men:	42%–52%	Adult women:	37%–47%
Newborn:	53%–65%								
Infant child:	30%–43%								
Adult men:	42%–52%								
Adult women:	37%–47%								

Method 31-10. Red Blood Cell Indices

The RBC indices are used to classify anemias based on the cell size and hemoglobin concentration of the red blood cells. These classifications are useful in the determination of the root cause of the anemia. The RBC distribution width (RDW) and mean platelet volume (MPV) values characterize the red blood cell and platelet populations, respectively. Whereas the RDW reflects the variation in size of red blood cell, the MPV represents the average size of platelets.

METHOD 31-10 Red Blood Cell Indices

Principle	The red blood cell indices include the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). The indices can be calculated from the red blood cell count, the hemoglobin, and hematocrit values. While the RDW is a calculation derived from the MCV, the MPV is determined from the arithmetic mean of the platelet histogram provided by the hematology analyzer. (See Chapter 32.)	
Specimen	EDTA-anticoagulated blood	
Procedure	See the required equipment and steps for this procedure at www.fadavis.com	
Interpretation	Determination of the MCV, MCH, and MCHC offers valuable information to characterize RBCs. The MCV reflects red blood cell size; thus, a higher MCV indicates macrocytic cells while a lower than normal MCV indicates microcytes. An MCV within reference values is normocytic. Meanwhile, the MCHC reflects the hemoglobin concentration. Thus, a low MCHC specifies hypochromic red blood cells while a normal MCHC is normochromic. There is an ongoing debate whether hyperchromic red blood cells should be a classification. Therefore, if a patient has red blood cells that have a low MCV and a low MCHC, the anemia would be classified as microcytic hypochromic. This type of anemia is most often associated with iron deficiency. Table 31-2 provides additional information for anemia classification based on the values of the indices. An elevated RDW may be seen on blood smears with varying degrees of anisocytosis. This parameter is useful in distinguishing iron-deficiency anemia (increased) from the hemoglobinopathies (normal). The MPV may be increased in thrombocytopenia or inflammatory diseases involving the cardiovascular and renal systems, rheumatoid diseases, and in some cancers, and decreased in systemic lupus erythematosus, colitis, and neoplastic processes. ¹⁰	
Limitations	The RDW may be the best indicator of anisocytosis in patients who have a dimorphic population or who have been transfused. In such cases, the MCV value can be normal with an increased RDW.	
Formulas	$\text{MCV} = \frac{\text{Hct}}{\text{RBC}} \times 10 = \text{fl}$ $\text{MCH} = \frac{\text{Hgb}}{\text{RBC}} \times 10 = \text{pg}$ $\text{MCHC} = \frac{\text{Hgb}}{\text{Hct}} \times 100 = \%$	
Reference Ranges	MCV	80–100 fL
	MCH	28–31 pg
	MCHC	32%–36%
	RDW	11.5%–14.5%
	MPV	7.4–10.4 fL

TABLE 31-2 Classification of Anemia

MCV (fL)	MCHC (%)	Classification
80–100	32–36	Normocytic, normochromic
<80	<32	Microcytic, hypochromic
>100	32–36	Macrocytic, normochromic

Method 31-11. Reticulocyte Counts

Reticulocytes are immature RBCs that contain remnant cytoplasmic ribonucleic acid (RNA) and organelles such as mitochondria and ribosomes. Reticulocytes are visualized by staining with supravital stains that precipitate the RNA and organelles, forming a filamentous network of reticulum (Fig. 31-6). On

**FIGURE 31-6** Photograph of a reticulocyte using new methylene blue stain.

Wright stain, the reticulocyte appears polychromatophilic or as a macrocytic blue-red cell. The reticulocyte is a means of assessing the erythropoietic activity of the bone marrow. Manual and automated methods were found to be equally precise and useful in clinical applications.¹¹

Method 31-11A. Reticulocyte Counts Using the Miller Disc

The Miller disc ocular method can be used to facilitate the counting of large numbers of RBCs in a more concise area.

TABLE 31-3 Maturation Time for Reticulocytes

Maturation Time	Hematocrit (%)
1 day	45
1.5 days	35
2 days	25
3 days	15

Standard Methods for Specific Anemias

The standard hematology methods for specific anemias include:

- Sickledex™
- Helena SPIFE® Alkaline Hemoglobin Electrophoresis
- Helena SPIFE® Acid Hemoglobin Electrophoresis
- Hemoglobin A₂ Determination
- Isoelectric Focusing
- Hemoglobin F Acid Stain
- Screening Test for Glucose-6-Phosphate Dehydrogenase Deficiency
- Staining for Heinz Bodies
- Screening Method for Detection of Red Cell Pyruvate Kinase

Method 31-12. Sickledex™ (aka sickle solubility testing)

Sickle cell disease is an autosomal recessive inherited mutation of the hemoglobin beta gene resulting in the production of hemoglobin S. This hemoglobin distorts the red blood cells into a crescent or sickle shape that affects red cell mobility through the smaller blood vessels. Additionally, there is an increased red blood cell turnover and persistent anemia.

METHOD 31-11 Reticulocyte Counts

Principle	Ribonucleoprotein present in young red cells (reticulocytes) react with supravital stains such as new methylene blue or brilliant cresyl blue, forming a blue precipitate within the red blood cell.										
Specimen	EDTA-anticoagulated blood										
Procedure	See the required equipment and steps for this procedure at www.fadavis.com										
Interpretation	<p>Increased reticulocyte counts often reflect a regenerative process and are expected in anemic conditions. Low reticulocyte counts are often associated with a hypoproliferative bone marrow.</p> <p>A corrected reticulocyte count compares the patient hematocrit value with a normal hematocrit value.</p> <p>The reticulocyte production index (RPI) is a result that accounts for the presence of "stress or shift" reticulocytes in the peripheral blood. These premature reticulocytes contain more filamentous reticulum and result in a longer maturation time in circulation. The RPI calculation considers the corrected reticulocyte count as well as the maturation time of the reticulocyte based upon patient hematocrit (see Table 31-3).</p>										
Limitations	Samples for manual reticulocyte should be analyzed within 72 hours of collection to avoid falsely decreased counts. ¹² Automated methods have exhibited stability up to 6 days when samples are refrigerated. ¹³										
Formulas	$\frac{\text{Number of reticulocytes} \times 100}{1,000 \text{ Red cells}} = \% \text{ reticulocytes}$ $\text{Reticulocyte (\%)} \times \frac{\text{Hct (\%)}}{45} = \text{corrected reticulocyte count}$ $\frac{\text{Reticulocyte (\%)} \times \text{RBC count (10}^{12}/\text{L)}}{100} = \text{absolute reticulocyte count}$ $\frac{\text{Reticulocyte (\%)} \times [\text{Hct (\%)} / 45]}{\text{Maturation time}} = \text{RPI}$										
Reporting Results	The reticulocyte count and the corrected reticulocyte are reported as percentages. The absolute reticulocyte count is reported as the number of reticulocytes in 10 ⁹ /L of blood.										
Reference Ranges	<table> <tr> <td>Reticulocyte Count</td><td>2.5%–6.0%</td></tr> <tr> <td>Newborn:</td><td>0.5%–2.0%</td></tr> <tr> <td>Adult:</td><td>24–80 × 10⁹/L</td></tr> <tr> <td>Absolute reticulocyte count:</td><td>3 or greater</td></tr> <tr> <td>RPI</td><td></td></tr> </table>	Reticulocyte Count	2.5%–6.0%	Newborn:	0.5%–2.0%	Adult:	24–80 × 10 ⁹ /L	Absolute reticulocyte count:	3 or greater	RPI	
Reticulocyte Count	2.5%–6.0%										
Newborn:	0.5%–2.0%										
Adult:	24–80 × 10 ⁹ /L										
Absolute reticulocyte count:	3 or greater										
RPI											

METHOD 31-11A Reticulocyte Counts Using the Miller Disc

Principle	Using the Miller ocular disc , reticulocytes and mature red cells are separated and counted in separate squares of the disc. Using a factor of 9, reticulocytes can be easily calculated.
Specimen	EDTA-anticoagulated blood
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	<p>Increased reticulocyte counts often reflect a regenerative process and are expected in anemic conditions. Low reticulocyte counts are often associated with a hypoproliferative bone marrow.</p> <p>The reticulocyte production index (RPI) is a result that accounts for the presence of "stress or shift" reticulocytes in the peripheral blood. These premature reticulocytes contain more filamentous reticulum and result in a longer maturation time in circulation. The RPI calculation considers the corrected reticulocyte count as well as the maturation time of the reticulocyte based upon patient hematocrit (Table 31-3).</p>
Limitations	Samples for manual reticulocyte should be analyzed within 72 hours of collection to avoid falsely decreased counts. ¹² Automated methods have exhibited stability up to 6 days when samples are refrigerated. ¹³
Formulas	$\frac{\# \text{ Reticulocytes} \times 100}{\text{Total RBC in smaller square} \times 9} = \% \text{ reticulocytes}$ $\text{Reticulocyte (\%)} \times \frac{\text{Hct (\%)}}{45} = \text{corrected reticulocyte count}$ $\frac{\text{Reticulocyte (\%)} \times \text{RBC count (10}^{12}/\text{L)}}{100} = \text{absolute reticulocyte count}$ $\frac{\text{Reticulocyte (\%)} \times [\text{Hct (\%)} / 45]}{\text{Maturation time}} = \text{RPI}$
Reporting Results	The <u>reticulocyte count</u> and the <u>corrected reticulocyte</u> is reported as a percentage. The absolute reticulocytes count is reported as the number of reticulocytes in $10^9/\text{L}$ of blood.
Reference Ranges	<p>Reticulocyte Count</p> <p>Newborn: 2.5%–6.0%</p> <p>Adult: 0.5%–2.0%</p> <p>Absolute reticulocyte count: $24\text{--}80 \times 10^9/\text{L}$</p> <p>RPI 3 or greater</p>

METHOD 31-12 Sickledex™

Principle	Red blood cells are lysed by a surfactant and the released hemoglobin is reduced by sodium hydrosulfite. While reduced hemoglobin A is soluble in this medium, hemoglobin S is insoluble and forms a turbid suspension in phosphate solution. Both sickle cell disease and sickle cell trait can be detected with the Sickledex™ test. ¹⁴
Specimen	EDTA-anticoagulated blood
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	<p>Positive: If hemoglobin S or another sickling hemoglobin is present, the solution will be turbid and the dark lines of the reading rack will not be visible when viewing through the tubes.</p> <p>Negative: If no sickling hemoglobin is present, the solution will be clear to slightly cloudy and the lines of the tube reading rack will be visible through the tubes. Both positive and negative reactions are illustrated in Figure 31-7.</p>
Limitations	<p>False positives are possible with increased hematocrit or leukocytosis. Hyperglobulinemia and hyperlipidemia may also appear turbid, resulting in a false positive reading. Patients with multiple myeloma or other plasma cell dyscrasias may yield false-positive results. Patients with hemoglobin variants such as hemoglobin C_{harlem} or hemoglobin E may yield positive reactions.</p> <p>False-negative results are possible in infants younger than 6 months of age or with elevated levels of hemoglobin F. Patients with hematocrit levels below 15% may appear falsely negative. A patient who has been recently transfused may yield false-negative results.</p>
Reporting Results	Results are reported as positive or negative.

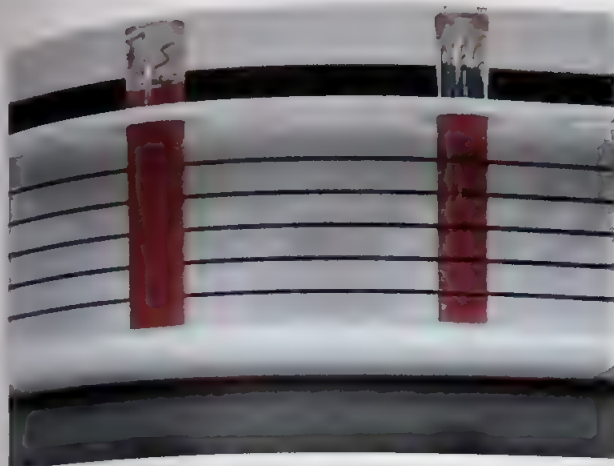


FIGURE 31-7 Positive (left) and negative (right) results of sickle solubility test.

Method 31-13. Helena SPIFE® Alkaline Hemoglobin Electrophoresis

Alkaline hemoglobin electrophoresis is the first confirmatory test following a positive sickle solubility test. It is also used to aid in identification of other abnormal hemoglobins seen in a variety of hemoglobinopathies and thalassemias.

Method 31-14. Helena SPIFE® Acid Hemoglobin Electrophoresis

Acid hemoglobin electrophoresis is performed following an inconclusive alkaline. This procedure is utilized to confirm the presence of abnormal hemoglobin S and/or C, as no other hemoglobins migrate in the same position with this process.

Method 31-15. Hemoglobin A₂ Determination

The quantification of hemoglobin A₂ is important in diagnosis of beta thalassemia. This test would most likely be considered a reference lab procedure, as most hospitals do not have the volume to warrant the equipment purchase.

Method 31-16. Isoelectric Focusing

Isoelectric focusing (IEF) is a technique used to separate molecules based on their isoelectric point. The isoelectric point is the pH at which a molecule carries no net electrical charge and becomes immobile in an electrical field. To have a sharp isoelectric point, a molecule must be amphoteric, meaning it must have both acidic and basic functional groups. Proteins and amino acids are common molecules that meet that requirement and therefore can be subjected to isoelectric focusing for separation.

Method 31-17. Hemoglobin F Acid Stain (Modified Kliehauer-Betke Test)

Hemoglobin F is composed of alpha and gamma proteins and is the prevalent form of hemoglobin present in fetal and newborn blood. Hemoglobin A consists of alpha and beta proteins and gradually becomes the prevalent hemoglobin at the age of 2. Hemoglobin F amounts level out below 2%

METHOD 31-13 Helena SPIFE® Alkaline Hemoglobin Electrophoresis

Principle Small samples of hemolysates prepared from packed cells are automatically applied to the SPIFE alkaline hemoglobin gel.¹⁵ The hemoglobins in the sample are separated by electrophoresis using an alkaline buffer and are stained with acid blue stain. The patterns are scanned on a densitometer and the relative percentage of each band is determined.

Specimen EDTA-anticoagulated blood

Procedure See the required equipment and steps for this procedure at www.fadavis.com

Interpretation The hemoglobin gels may be inspected visually for the presence of hemoglobin bands. The Helena Hemo controls provide a marker for the band identification. The controls AA₂ and AFSC Hemo Control should be used as markers for the location of hemoglobin bands. Hemoglobin electrophoresis on cellulose acetate and citrate agar is depicted in Figure 31-8. In addition, the relative percentage of each hemoglobin band can be determined by scanning the dried gels in the densitometer using a 595-nm filter.

Limitations Citrate agar electrophoresis may be necessary to confirm abnormal hemoglobins, whereas anion-exchange column chromatography is a more accurate method for quantitating HgbA₂.

Reporting Results Hemoglobin relative concentration is reported as a percent compared with the hemoglobin proteins detected in the analyzed sample.

Reference Ranges	Birth:	100% Hgb F
	Adult:	98% Hgb A
		3.5% Hgb A ₂
		<2% Hgb F

after the age of 10. Identification of fetal red blood cells in the peripheral blood of a pregnant woman forewarns possible maternal antibody development against the fetus. In these cases, antibody development can be prevented through RhoGAM® administration.

Method 31-18. Screening Test for Glucose-6-Phosphate Dehydrogenase Deficiency

Glucose-6-Phosphate Dehydrogenase deficiency is a genetic mutation of the G6PD gene. G6PD normally protects RBCs from harmful by-products of cellular metabolism. Decreased or absence of G6PD results in varying levels of hemolytic anemia. Glucose-6-phosphate dehydrogenase deficiency remains the most prevalent enzyme deficiency in the world.²¹

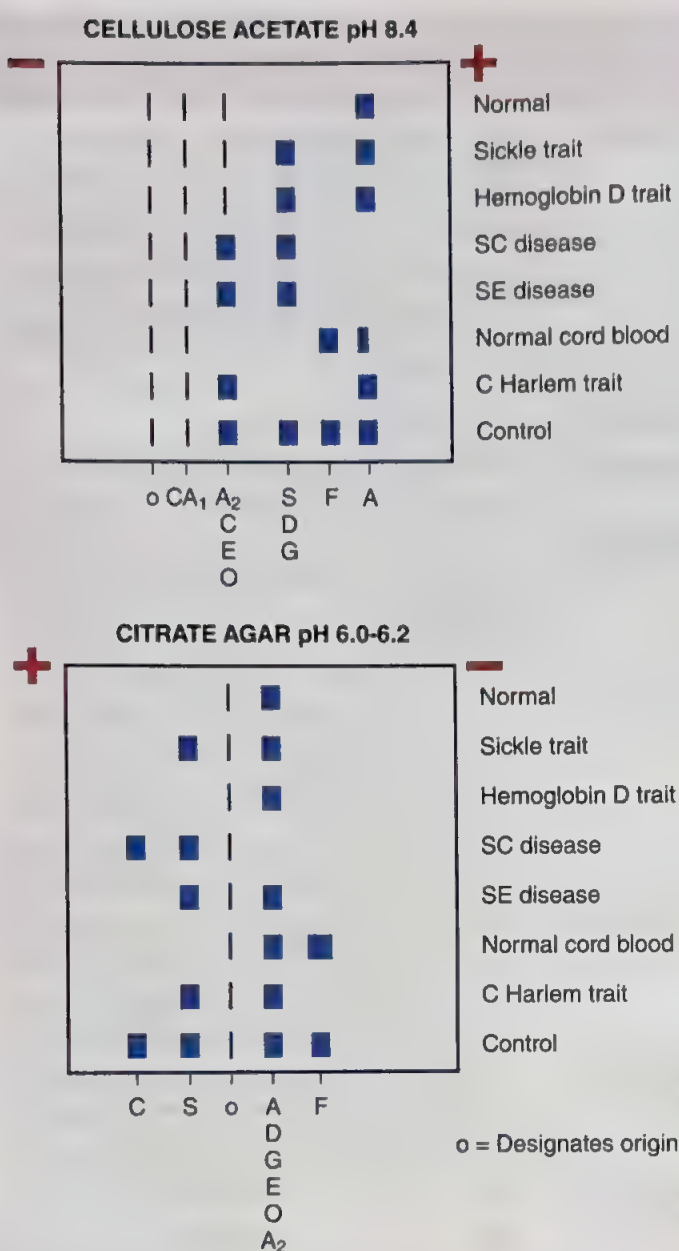


FIGURE 31-8 Comparative hemoglobin electrophoresis. Hemoglobin electrophoresis on cellulose acetate and citrate agar, indicating patterns of mobility. The width of the band is not indicative of hemoglobin concentration.

METHOD 31-14 Helena SPIFE® Acid Hemoglobin Electrophoresis

Principle	Very small samples of hemolysates prepared from packed cells are automatically applied to the SPIFE Acid Hemoglobin gel. The various hemoglobin proteins in the sample are separated by electrophoresis using a citrate buffer and stained with acid blue stain.	
Specimen	EDTA-anticoagulated blood	
Procedure	See the required equipment and steps for this procedure at www.fadavis.com	
Interpretation	The hemoglobin gels should be inspected visually for the presence of abnormal hemoglobin bands (see Fig. 31-8). Glycated hemoglobin migrates with HbF. The Helena AFSC Hemo Control provides a marker for band identification. This control should be run on each SPIFE Acid Hemoglobin gel. The control verifies all phases of the procedure and acts as a marker to aid in the identification of the hemoglobins in the unknown samples.	
Limitations	Anion-exchange column chromatography is the most accurate method for quantitating HgbA ₂ . Low levels of HgbF may be accurately quantitated using radial immunodiffusion.	
Reporting Results	Each hemoglobin fraction is reported as a percent of the total hemoglobin protein in the sample.	
Reference Ranges	Birth:	100% Hgb F
	Adult:	98% Hgb A 3.5% Hgb A ₂ <2% Hgb F

METHOD 31-15 Hemoglobin A₂ Determination

Principle	Although hemoglobin A ₂ makes up a mere 3% of total adult hemoglobin, quantification of Hgb A ₂ is an important approach in the diagnosis of beta thalassemia trait. ¹⁷ The Helena Beta-Thal-HbA ₂ protocol is a chromatographic approach to identify and differentiate various hemoglobin proteins. ¹⁸ In this methodology, an anion-exchange resin is composed of cellulose covalently coupled to small positively charged molecules. The positively charged cellulose attracts negatively charged molecules. Proteins, such as the hemoglobins, contain many positive and negative charges due to the ionizing properties of the component amino acids. In the anion-exchange chromatography of HbA ₂ , buffer and pH levels are controlled to cause different hemoglobins to possess different net negative charges. These negatively charged proteins are attracted to the positively charged cellulose and bind accordingly. After binding, the proteins are removed selectively from the resin by altering the pH or ionic strength of the elution buffer. Because of the pH of the resin and the ionic strength of the HbA ₂ developer, HbA ₂ does not bind to the positively charged cellulose and is eluted as the developer moves through the column. The other normal and most abnormal hemoglobins are retained by the resin. The HbA ₂ fraction is compared with a total hemoglobin fraction by determining the absorbance of each using a spectrophotometer and then calculating the percentage of HbA ₂ .
Specimen	EDTA-anticoagulated blood
Procedure	See the required equipment and steps for this procedure at www.fadavis.com

METHOD 31-15 Hemoglobin A₂ Determination—cont'd**Interpretation**

Results of HbA₂ should be interpreted in conjunction with patient history, total hemoglobin values, and other clinical and lab findings. Any value between 3.5% and 8% is considered indicative of beta (β)-thalassemia trait. Values above 8% indicate the presence of additional hemoglobin variants such as HbC, E, O, D, G, S, or S-G hybrid.

Limitations

Failure to completely resuspend the contents of the column may cause slow flow and erroneous results. Time must be allowed after resuspension for the formation of a distinct interface between the resin and supernatant. Any trapped bubbles may be removed with a Pasteur pipet.

The bottom tip closure must be removed immediately after resuspension. Resuspension must be repeated if the column is allowed to sit with the bottom closure in place after resuspension. Failure to do so may cause slow flow and erroneous values.

As soon as the resin repacks, the remaining supernatant must be aspirated and discarded.

To avoid back pressure in the column, do not remove the bottom tip closure before removing the top cap closure. Any bubbles trapped in the column resin may slow or stop the flow rate, leading to erroneous results.

If the developer ceases to flow through the column, the column must be discarded and the quantification repeated with a fresh column.

Any disturbance of the resin during the procedure may cause erroneous results. No more than 5 minutes should elapse from the time the column stops flowing until the developer is added so that the resin does not dry out.

Some abnormal hemoglobin proteins (HbS, C, E, O, E, G, S-G hybrid) are eluted with HbA₂ in this method. The presence of abnormal hemoglobin proteins should be confirmed by electrophoretic techniques. HbF does not interfere with this method.

Reporting Results

The amount of hemoglobin A₂ present is reported as a percentage.

Reference Ranges

HbA₂ range = 2.2% to 3.3%

METHOD 31-16 Isoelectric Focusing**Principle**

At a pH below the isoelectric point (pI), proteins carry a net positive charge. Above the pI, they carry a net negative charge. The pH of an electrophoretic gel is determined by the buffer used for that gel. If the pH of the buffer is above the pI of the protein being run, the protein will migrate to the positive pole. If the pH of the buffer is below the pI of the protein being run, the protein will migrate to the negative pole of the gel. If the protein is run with a buffer pH that is equal to the pI, it will not migrate at all. By using ampholyte buffers in the pH range of 6 to 8, the separation and identification of abnormal hemoglobins are possible based on migration of proteins based on their isoelectric points.¹⁹

Specimen

EDTA-anticoagulated blood or heparinized blood

Procedure

See the required equipment and steps for this procedure at www.fadavis.com

Interpretation

HbE and HbO_{Arab} migrate slightly anodal to HbA₂. HbG-Philadelphia and HbD-Los Angeles are separated from HbS as is Hb_{Lepore}. HbA and HbF are clearly separated from each other, allowing identification between sickle cell trait, sickle cell anemia, and HbS/β-thalassemia (Fig. 31-9). The AFSC Control and AA₂ control should be run with each gel. Dilute the AFSC Control 1:2 with hemolysate reagent before use.

Limitations

HbG_{Calverton} and HbG_{Marick} cannot be distinguished from HbS.

Hb_{Hennepin}, Hb_{Bethesda}, and Hb_{Bagham} cannot be distinguished from HbA.

HbE, HbC_{Marion}, HbO_{Arab}, and Hb_{Kato} cannot be separated.

HbN_{Baltimore} and HbL_{Texas} cannot be separated.

HbCC cannot be distinguished from HbC/β-thalassemia, and HbEE cannot be distinguished from HbE/β-thalassemia.

If the gels exhibit drying under low ambient humidity, bands will be skewed and exhibit uneven migration. A band will migrate in an arched manner if the concentration of hemoglobin is too high. A sample application of 1 μL should rectify the problem.

Bands from fresh whole blood may electrophorese at a slightly slower rate than the control. Older samples may electrophorese at a slightly faster rate than the control. If controls do not perform as expected, test results should be considered suspect or invalid.

Reporting Results

Hemoglobin variants are identified by their relative location in comparison with controls. All hemoglobin identified are reported as present.

Reference Ranges

In order of abundance from most abundant to least

Newborn;

HbF, HbA

Infant;

HbA, HbF, HbA₂

Adult;

HbA, HbA₂, HbF

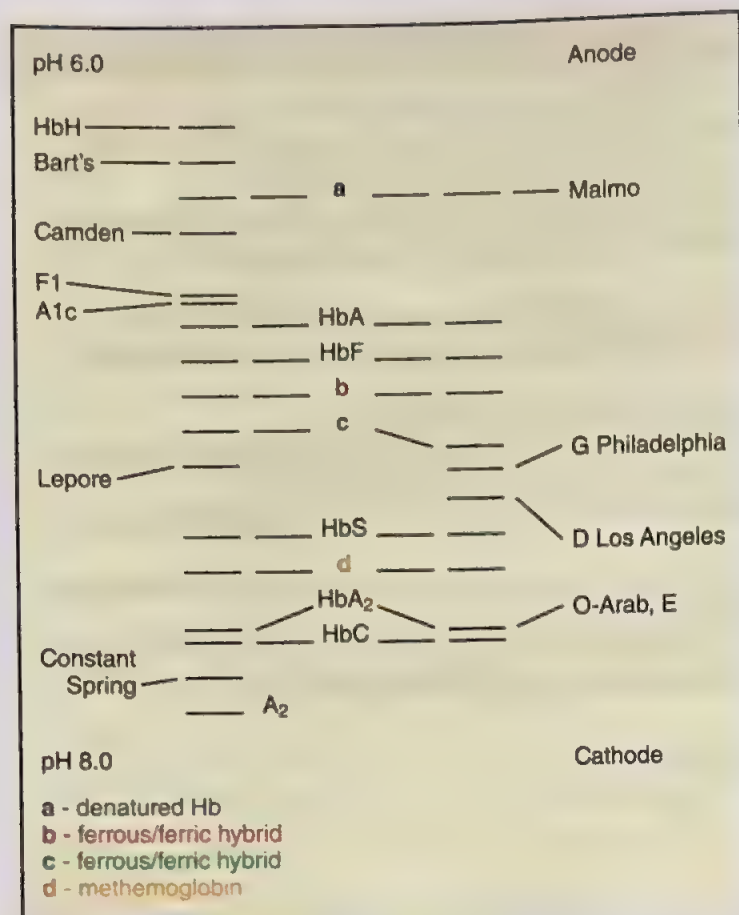


FIGURE 31-9 Schematic of isoelectric focusing pattern. (Helena Laboratories, Beaumont, TX.)

METHOD 31-17 Hemoglobin F Acid Stain (Modified Kliehauer-Betke Test)

Principle	Red cells containing fetal hemoglobin are resistant to acid elution, whereas red cells containing hemoglobin A will appear as ghost cells on exposure to acid.
Specimen	EDTA-anticoagulated blood
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	Any value not reported as zero (0) is abnormal and indicates a fetal-maternal hemorrhage. ²⁰
Limitations	Hematologic disorders (e.g., thalassemia) may produce increased levels of fetal cells. The degree of elution of adult hemoglobin may vary from patient to patient. Lymphocytes may take up stain in varying degrees, albeit less than fetal cells.
Formulas	To determine the percentage of fetal cells to adult cells, divide the total number of fetal cells counted by the number of adult cells counted. For example: If 25 fetal red blood cells and 4,000 adult cells are counted: $25/4,000 \times 100 = 0.6\% \text{ fetal cells.}$
Reporting Results	Fetal cells are reported as a percentage of fetal cells in maternal blood.
Reference Ranges	Fetal cells in maternal blood = 0.

METHOD 31-18 Screening Test for Glucose-6-Phosphate Dehydrogenase Deficiency

Principle

Glucose-6-phosphate (not fluorescent) is oxidized to 6-phosphogluconate (fluorescent), and NADP is reduced to NADPH in the presence of glucose-6-phosphate dehydrogenase (G6PD) from the red cell hemolysate. The red cells also contain 6-phosphogluconate which reduces more NADP. The NADPH when activated by UV light, produces a vivid fluorescence.⁵ Spot testing for fluorescence is a common methodology.²¹

Specimen	Venous blood
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	Classify the G6PD activity of each sample by the level of fluorescence observed (Fig. 31-10). <ul style="list-style-type: none"> • Normal G6PD activity: G6PD activity exhibits moderate to strong fluorescence after 5 minutes and strong fluorescence after 10 minutes. • Intermediate G6PD activity: Weak fluorescence after 5 minutes and moderate fluorescence after 10 minutes. • Deficient G6PD activity: Weak or no fluorescence after 5 and 10 minutes.
Limitations	Blood with a hematocrit below 25% is contraindicated. If anemic blood samples are tested, adjust the hematocrit to within 40% to 45%.
Reporting Results	G6PD activity is semi-quantitatively reported as Normal, Intermediate, or Deficient.
Reference Ranges	G6PD activity should be normal.

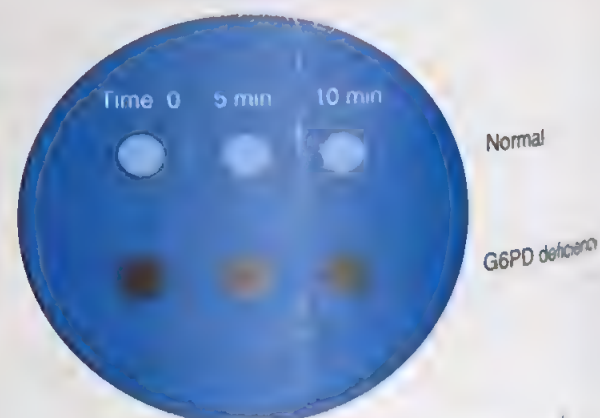


FIGURE 31-10 The G6PD fluorescent spot test is a peripheral blood in vitro screening test. The results are viewed under ultraviolet light at 365nm wavelength. A normal patient will exhibit fluorescence at all time points, and a G6PD deficient sample will not fluoresce.

Method 31-19. Staining for Heinz Bodies

METHOD 31-19 Staining for Heinz Bodies

Principle	Heinz bodies are denatured hemoglobin precipitated in the RBC and attached to the RBC membrane. The presence of Heinz bodies is evidence of oxidative damage to hemoglobin and is associated with the most common RBC enzymopathy, G6PD. ²³ Heinz bodies are not visible with Wright's stain but can be visualized using supravital stain (crystal violet) and phase microscopy (Fig. 31-11).
Specimen	EDTA-anticoagulated blood
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	Heinz bodies are precipitated hemoglobin and appear as irregular, refractile, purple inclusions, 1 to 3 μm in diameter, located on the periphery of the cell. ²⁴ They may even seem to be outside the cell. The presence of Heinz bodies indicates oxidative denaturation of hemoglobin. Reticulocytes are not stained by this technique.
Limitations	Heinz body stains performed with supravital stains necessitate differentiation between Heinz bodies and reticulocytes.
Reporting Results	Heinz bodies are reported as present or none seen
Reference Ranges	No Heinz bodies observed.



FIGURE 31-11 Heinz bodies

Method 31-20. Screening Method for Detection of Red Cell Pyruvate Kinase

Pyruvate kinase is an essential enzyme in glycolysis that produces energy in red blood cells, and therefore, decreased levels result in shortened red blood cell survival. Decreased levels are attributed to inherited autosomal recessive mutations in the PKHR gene, producing a chronic hemolytic anemia.²⁵

METHOD 31-20 Screening Method for Detection of Red Cell Pyruvate Kinase

Principle	A phosphate group is transferred to ADP, forming pyruvate and ATP in the presence of red cell pyruvate kinase. Lactate dehydrogenase in the red cell hemolysate catalyzes the reduction of pyruvate to lactate with the resulting oxidation of diphosphopyridine nucleotide (DPNH) to diphosphopyridine (DPN). DPNH has the property of fluorescence, while DPN does not fluoresce under UV light.
Specimen	EDTA-anticoagulated blood
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	The first spot from a normal blood sample will fluoresce brightly, but the second spot will not fluoresce. Red cells, deficient in pyruvate kinase, fluoresce in both spots. ⁵
Limitations	All frozen reagents are stable for 1 month. Reaction mixture is stable at room temperature for 7 hours. EDTA blood samples are stable for 5 days at room temperature. False normal results may be obtained in samples with incomplete removal of leukocytes or after transfusion with normal donor cells.
Reporting Results	Normal is reported when 1 of 2 spots fluoresces. Low or abnormal is reported when both spots fluoresce. Genetic testing for the PKHR gene is suggested with low and abnormal results.
Reference Ranges	Normal

Nonspecific Tests of Inflammation

Nonspecific hematology tests for inflammation include the Westergren Erythrocyte Sedimentation Rate and the Alifax™ Test 1 Automated Erythrocyte Sedimentation Rate Analyzer.

Method 31-21. Westergren Erythrocyte Sedimentation Rate

The erythrocyte sedimentation rate (ESR) is a nonspecific test used to detect illnesses associated with acute and chronic infection, inflammation and tissue necrosis or infarction.

METHOD 31-21 Westergren Erythrocyte Sedimentation Rate

Principle	The manual Westergren ESR measures the settling of erythrocytes in diluted human plasma over a specified time period and represents the gold standard for sedimentation rate analysis. ²⁶ There are three stages in the sedimentation rate of erythrocytes. Red blood cells initially aggregate in the first stage of the sedimentation rate followed by the second stage involving precipitation. Finally, the precipitated aggregates of red blood cells become packed at the bottom of the sedimentation tube. This ESR numeric value is determined (in millimeters) by measuring the distance from the bottom of the surface meniscus to the top of erythrocyte sedimentation in a vertical column containing diluted whole blood that has remained perpendicular to its base for 60 minutes. Various factors affect the ESR, such as RBC size and shape, plasma fibrinogen, and globulin levels, as well as mechanical and technical factors. The ESR is directly proportional to the RBC mass and inversely proportional to plasma viscosity. In normal whole blood, RBCs do not form rouleaux; the RBC mass is small and therefore the ESR is decreased (cells settle out slowly). In abnormal conditions when RBCs can form rouleaux, the RBC mass is greater, thus increasing the ESR (cells settle out faster). The Westergren tube is 30 cm long, 2.5 mm in diameter, and calibrated in mm from 0 to 200.	
Specimen	EDTA-anticoagulated blood	
Procedure	See the required equipment and steps for this procedure at www.fadavis.com	
Interpretation	Various diseases can yield an increased ESR, such as rheumatoid arthritis, multiple myeloma, anemia, and inflammatory diseases, among others. Pregnancy can also cause an increased ESR. Although RBCs normally have negative charges that tend to repel each other, plasma proteins possess positive charges. Increased levels of plasma proteins negate the negative charge of red blood cells, allowing less space between cells. An increased ESR is often associated with abnormal levels of circulating acute phase proteins such as fibrinogen and immunoglobulins that are associated with systemic diseases. Quantitative red blood cell disorders such as anemia and polycythemia can affect ESR values as well as hemoglobinopathies. Sickle cell anemia can cause low ESR results. Abnormal red cell morphology may be associated with a decreased ESR. ²⁷	
Limitations	Low results can occur when the collected specimen is allowed to stand for more than 3 hours. The Westergren tube must be situated perfectly vertical as any tilt can cause invalid results.	
Reporting Results	Sedimentation rates are reported as the amount in mm the red blood cells settle in 1 hour (mm/hr).	
Reference Ranges	Adult men:	0–15 mm/h
	Adult women:	0–20 mm/h

Method 31-22. Alifax® Erythrocyte Sedimentation Rate Analyzer

The Sysmex Alifax® Sedimentation Rate Analyzer uses capillary photometry technology to provide results in 20 seconds. The principle methodology measures the kinetics of red blood

cell aggregation taking 1,000 measurements over 20 seconds. Comparisons between the classic Westergren Sedimentation Rates and Capillary Sedimentation Rates exhibited acceptable correlation.²⁸

METHOD 31-22 Alifax® Erythrocyte Sedimentation Rate Analyzer

Principle	The instrument uses a closed system to aspirate 175 µL of blood into a capillary tube. The sample is centrifuged at 20 g, and the RBCs present at the capillary level are assessed via an infrared ray at 950 nm 1,000 times over 20 seconds. This information is applied to a sedimentation curve and the correlating Westergren value is mathematically obtained. The results are recorded as mm per hour. CLSI guidelines recognize the Alifax System as an acceptable alternative to the Westergren ESR method.	
Specimen	EDTA blood sample, minimum 1.5 mL	
Procedure	See the required equipment and steps for this procedure at www.fadavis.com	
Interpretation	The Alifax® will automatically scan the samples and print the results when analysis is complete. Any error codes will be printed on the ticket with the results. Latex controls are available to verify instrument accuracy.	
Limitations	The K2/K3 EDTA tube must have a minimum of 1.5 mL and should not exceed 60% filled volume. The sample should not contain clots/aggregates, be grossly hemolyzed, or drawn above an IV. Optimal time from collection to analysis is 4 hours. Refrigerated samples are stable up to 24 hours but must be allowed to return to room temperature for approximately 15 minutes before analysis. The reportable range is 2–120 mm/h. ²⁹	
Reporting Results	Sedimentation rates are reported in mm/hr.	
Reference Ranges	Adult men:	0–20 mm/h
	Adult women:	0–15 mm/h

CASE STUDY 31-1

A 73-year-old woman diagnosed with multiple myeloma is currently on melphalan and prednisone. Her CBC revealed the following:

		Reference Ranges
WBC	$3.6 \times 10^9/L$	$4.8-5.4 \times 10^9/L$
RBC	$3.2 \times 10^{12}/L$	$4.2-5.4 \times 10^{12}/L$
Hgb	12.1 g/dL	12-16 g/dL
Hct	36%	37%-47%
MCV	100 fL	80-100 fL
MCH	30 pg	28-31 pg
MCHC	31%	32%-36%
Platelets	—	

Manual differential:

Plasma cells	5%	0
Lymphocytes	30%	20%-44%
Segs	50%	50%-70%
Bands	5%	2%-6%
Monocytes	7%	2%-9%
Eosinophils	3%	0%-4%
NRBCs	12/100 WBCs	0

QUESTIONS

1. What is the corrected WBC count?
2. Based on the corrected WBC count, what are the absolute counts for each white blood cell population?
3. Based on this patient's results, is there an anemic state? If so, which results indicate this?

ANSWERS:

1. Using the formula from this chapter, the corrected WBC count can be calculated as $3.3 \times 10^9/L$.
2. Using the formula from this chapter, the absolute counts for each cell identified calculate as follows:
 - Plasma cell: $0.17 \times 10^9/L$
 - Lymphocytes: $0.99 \times 10^9/L$
 - Segs: $1.65 \times 10^9/L$
 - Bands: $0.17 \times 10^9/L$
 - Monocytes: $0.23 \times 10^9/L$
 - Eosinophils: $0.10 \times 10^9/L$
3. The low RBC count, slightly low Hct, and presence of NRBCs may indicate a slight anemia, although the Hgb is normal.

REVIEW QUESTIONS

1. Twenty microliters of blood are drawn into a Leuko-Tic system for a WBC count. Fifty cells are counted on one side and 52 on the other side of the hemocytometer (4 large squares). Calculate the WBC count.
 - a. $12.5 \times 10^9/L$
 - b. $12.7 \times 10^9/L$
 - c. $12.0 \times 10^9/L$
 - d. $3.0 \times 10^9/L$
2. Which of the following anticoagulants is utilized for hematology analysis?
 - a. Heparin
 - b. EDTA
 - c. Sodium citrate
 - d. No anticoagulant is utilized
3. Which of the following conditions is indicated with the erythrocyte sedimentation rate (ESR)?
 - a. Inflammation
 - b. Anemia
 - c. Thrombocytopenia
 - d. Polikilocytosis
4. Which hematology analysis is best for aiding in the diagnosis of sickle cell anemia?
 - a. Hemoglobin electrophoresis
 - b. ESR
 - c. Kliehauer-Betke stain
 - d. Reticulocyte count
5. Which of the following is the greatest risk for preanalytical error when collecting a capillary specimen for hematology analysis?
 - a. Lipemia
 - b. Leukocytosis
 - c. Dilution of cellular components
 - d. Thrombocytopenia
6. Eosin Y stains which cellular component?
 - a. Nucleus
 - b. Cytoplasm
 - c. Chromatin
 - d. Vacuoles
7. A patient has a low absolute lymphocyte count. Which of the following represents an appropriate count for this patient?
 - a. $3.2 \times 10^9/L$
 - b. $1.7 \times 10^9/L$
 - c. $2.3 \times 10^9/L$
 - d. $0.9 \times 10^9/L$
8. A patient presents with an MCV of 78 fL and an MCHC of 33%. How would this anemia be classified?
 - a. Microcytic, normochromic
 - b. Normocytic, normochromic
 - c. Microcytic, hypochromic
 - d. Normochromic, hyperchromic

Continued

REVIEW QUESTIONS—cont'd

9. A sample with thrombocytopenia, leukocytopenia, and reticulocytopenia correlates with:
- Hemolyzed sample
 - Lipemic sample
 - Contaminated with iv sample
 - Instrument error

See answers at the back of this book.

SUMMARY CHART

- The most common source of erroneous results is preanalytical errors.
- Specimens for hematology analysis should be whole blood samples in EDTA anticoagulant.
- The most common site for a capillary stick in infants is the plantar surface of the heel.
- The hemocytometer counting chamber is used for manual cell counting. The counting chamber has a total area of 9 mm²; the four corner primary squares are used for counting leukocytes, the center square for platelets, and the four corner and center squares of the primary square for counting erythrocytes.
- Cells for peripheral blood smear review are stained with Wright stain.
- Leukocytes are reported in relative and absolute counts.
- Reticulocytes are immature RBCs that contain remnant cytoplasmic RNA and can be visualized by supravital stains such as new methylene blue.
- The normal reticulocyte count in adult and newborn blood is 0.5% to 2.0% and 2% to 6%, respectively.
- A reticulocyte production index (RPI) of 3 or more represents an adequate response of erythropoiesis by the bone marrow to states of anemia.
- The hemoglobin concentration, hematocrit percentage, and red cells indices (RDW, MCV, MCH, and MCHC) can all be used to indicate anemia.
- Hematocrit is defined as the volume occupied by erythrocytes in a given volume of blood and is usually expressed as a percentage of the volume of whole blood.
- The normal hematocrit for men is 42% to 52%; for women, it is 37% to 47%.
- The hematocrit is usually three times the hemoglobin value.
- A corrected white blood cell count is calculated by multiplying the WBC/mm³ by 100 and dividing by 100 plus the number of nucleated RBCs per 100 WBCs.
- The normal hemoglobin value for a man is 14 to 18 g/dL; for a woman, it is 12 to 16 g/dL.
- Electrophoresis is defined as the movement of charged particles in an electric field; hemoglobin electrophoresis relies on the premise that abnormal hemoglobins migrate at a fixed pH.
- Isoelectric focusing (IEF) is used for separating and identifying abnormal hemoglobins based on their specific isoelectric points (pI).
- The solubility test for hemoglobin S is based on the deoxyhemoglobin properties of HbS; when exposed to high-molarity phosphate buffer, HbS will precipitate out of solution.
- The acid elution test for HbF is based on the principle that the HbF is resistant to acid elution and will retain the pink stain; hemoglobin A will be eluted from the red cell and appear colorless (ghost cells).
- Heinz bodies are denatured hemoglobin precipitated in the RBC and attached to the red cell membrane; they are visible by utilization of supravital stain such as crystal violet, and their presence can indicate anemia caused by G6PD deficiency.
- The osmotic fragility test is a measure of the surface-to-volume ratio of the red cell.

Principles of Automated Differential Analysis

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CHAPTER OUTLINE

Specimen Evaluation by Cell Volume and VCS Technology: DxH Analyzer Series, Beckman Coulter®

Red Cell Analysis
Platelet Analysis
Leukocyte Analysis
Reticulocyte Analysis
Nucleated Red Blood Cell Detection
Abnormal Flags
Body Fluid Analysis
Additional Parameters

Specimen Evaluation by Light Scattering and Cytochemical Analysis: ADVIA® Hematology Systems, Siemens Healthcare Diagnostics

Red Cell Analysis
Platelet Analysis
Leukocyte Analysis
Reticulocyte Analysis

Nucleated Red Blood Cell Detection
Abnormal Flags
Cerebrospinal Fluid Analysis

Specimen Evaluation With Hydrodynamic Focusing, RF/DC Technology, and Fluorescent Flow Cytometry: The Sysmex XN and XN-L Series Hematology Analyzers

Red Cell Analysis
Platelet Analysis
Leukocyte Analysis
Reticulocyte Analysis
Nucleated Red Blood Cell Detection
Abnormal Flags
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Specimen Evaluation by Multi-Angle Polarized Scatter Separation (MAPSS™) Technology: Abbott Alinity h Series

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Digital Morphology Analyzers: CellaVision Systems

Quality Assurance and Quality Control Measures for Automated Complete Blood Count Instruments
Quality Control Procedures
Quality Assurance Measures
Result Verification and Decision Rules
Summary Chart
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Review Questions
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LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 32-1 Explain the measurement principles used by modern hematology analyzers.
- 32-2 Interpret patient data generated by hematology analyzers, including hemogram parameters, red blood cell histograms, platelet histograms, and leukocyte histograms/scattergrams.
- 32-3 Discuss the use of automated morphology analyzers to evaluate peripheral blood and body fluid differentials.

- 32-4 Assess the importance and frequency of quality control and quality assurance for automated hematology analyzers.
- 32-5 Explain when to accept an automated differential or perform a manual differential
- 32-6 Identify circumstances that could contribute to spurious or artifactual results

Since 1954, with the inception of the first automated hematology analyzer, the Coulter Model A, the use of advanced technology to perform the routine complete blood count (CBC) has not only increased the efficiency of the hematology laboratory but has also improved medical decision-making. The **Coulter Principle** for enumerating and sizing particles in a solution is based upon measuring the change in impedance, or change in electrical resistance, that is created by a cell as it passes through an aperture. Cells are suspended in a solution

and directed to pass through the aperture to be measured. As the cell passes through the aperture, it creates a pulse. The number and size of pulses created can be used to quantitate and size cells present in a patient sample. The Coulter Principle is considered the gold standard for cell counting, and many modern hematology analyzers employ this technology in combination with the measurement of optical light scatter, cytochemistry, or fluorescence to quantitate, size, and characterize cells.

In addition to determining the red blood cell (RBC), platelet (PLT), and white blood cell (WBC) counts, modern hematology analyzers also provide accurate measurement of hemoglobin (HGB), hematocrit (HCT), and the RBC indices, which include mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). A red cell distribution width (RDW) and mean platelet volume (MPV) are also determined as part of the CBC. In addition, a five-part differential reporting the percentage and absolute numbers of neutrophils, lymphocytes, monocytes, eosinophils, and basophils is standard. Current instruments can also differentiate immature granulocyte populations, enumerate nucleated red blood cells, quantitate reticulocytes, and perform body fluid cell counting.

This chapter provides an overview of the technology and capabilities of current automated hematology instrumentation available from Beckman Coulter, Siemens Healthcare Diagnostics, Sysmex Corporation, Abbott Hematology, and CellaVision AB. The chapter concludes with a discussion of **quality assurance** and **quality control** measures associated with automated hematology instruments and case study presentations.

Although the discussion of analyzers in this chapter are limited to five manufacturers, there are several other automated hematology analyzers that provide complete blood counts, cell differentials, and digital morphology on the market. Owing to the abundance of manufacturers and publication delay factors, it is not feasible to identify and individually discuss all these systems. Information, instrument manuals, and descriptions of upgraded models are usually available from the manufacturers. The authors of this text do not endorse any particular manufacturer or model.

Specimen Evaluation: Cell Volume and VCS Technology, DxH Analyzer Series, Beckman Coulter®

Beckman Coulter hematology analyzers utilize Coulter® principle technologies to enumerate and size red blood cells, white blood cells, and platelets based on the measurable change in **impedance** that is created as a particle passes through an aperture concurrently with an electrical pulse. WBC differential analysis uses **VCS technology**, which is a combination of cell volume, conductivity, and light scatter measurements to differentiate white blood cells and enumerate nucleated red blood cells (NRBCs) (Table 32-1).

The original Coulter VCS technology is a single-channel analysis method that uses three independent energy sources to probe approximately 8,000 cells in their near-native state. **Cell volume (V)** is based on the Coulter Principle of electrical impedance to measure the size of the cells. **Conductivity (C)** measurements, also called opacity, are made using a high-frequency electromagnetic probe that reflects the nuclear, granular, and chemical properties of cells. This opacity measurement allows the instrument to calculate the nuclear/cytoplasmic ratio—a feature useful in distinguishing variant lymphocytes from normal lymphocytes. Conductivity measurements aid in differentiation of cells with different

TABLE 32-1 Measurements Associated With Coulter's VCS Technology

Method	Measurement
VCS	Approximately 8,000 cells in their near-native state pass through a sensing zone one particle at a time. <i>Volume, Conductivity, and Scatter</i> are measured by independent sources.
Volume (V)	As each cell passes through the sensing zone, the volume displaced is measured using low-frequency (DC) impedance.
Conductivity (C)	High-frequency (RF) currents penetrate the cell membrane. Changes in conductivity provide information about the size and internal structure of the cells. Conductivity is also referred to as opacity.
Scatter (S)	When an unstained cell is struck by a laser beam, light is both absorbed and scattered in every direction. Sensors collect light-scatter signals from multiple angles to obtain information about cell surface structure and cytoplasmic granularity.

Source: Adapted from the Beckman Coulter Cellular DxH Series Principles of Operation, with permission.

internal structures but of similar size such as lymphocytes and basophils. **Light scattering (S)** from a focused elliptical light beam from a helium-neon laser provides additional information about cell granularity, nuclear lobularity, and cell surface characteristics, and is particularly useful for identifying eosinophils.

Application of advanced VCS 360 technologies and the advanced Coulter® Principle in combination with scalable automation solutions are used by the latest generation Beckman Coulter® DxH Series (DxH 900, DxH690T, DxH Slidemaker and Stainer, DxH 560, DxH 520, and DxH 500) fully automated CBC and differential analyzers. These technologies allow high-resolution cell analysis, provide improved characterization of difficult sample types, and extended analytical ranges for cytopenic patients. The top-of-the-range DxH 900 completely automate not only the analytical testing steps but also many of the preanalytical and postanalytical testing steps including sample sorting and handling, automatic validation of results, and selective slide-making based on laboratory workflow rules. The DxH 690T is designed for medium-throughput laboratories and incorporates many of the analytical features found in the 900 Series analyzers. Previously released high-medium throughput analyzer models utilizing VCS and Coulter® Principle technologies include LH series (750/780/800), DxH 600/800, Gen•ST™, HmX, and MaxM™. The new DxH 560/520/500 series analyzers are designed to serve low-volume facilities, replacing the original AcT-Diff series.

Red Cell Analysis

The red cell histogram depicts particles with a volume of greater than 36 femtoliters (fL) and less than or equal to 360 fL. The RBC histogram displays particles with a volume

greater than 24 fL, but the mean corpuscular volume (MCV) is calculated from the area under the curve of the **RBC histogram**, depicted in Figure 32-1. Deviations in the shape and position of the RBC histogram indicate changes in RBC size, shape, or both. An RBC distribution that is shifted to the left indicates the presence of microcytosis as seen in iron deficiency anemia or thalassemia minor. Conversely, a shift to the right such as in folate deficiency and liver disease indicates the presence of macrocytes.

The availability of red cell parameters such as RBC, HGB, HCT, MCV, mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), in conjunction with information derived from RBC histograms, may provide valuable information for assessing red cell disorders. The red cell distribution width (RDW) represents a parameter that quantifies the degree of relative RBC anisocytosis or variation in cell size. The DxH series provides two measurements of RDW: (1) as a coefficient of variation (CV) of the MCV, known as RDW, and (2) RDW-SD, which is measured by calculating the width in fL at the 20% height level on the RBC curve, as in:

$$\text{RDW (CV)} = \frac{\text{SD}}{\text{Mean}} \times 100$$

Normal range = 11.5%–14.5%

RDW-SD is measured by calculating the width in fL at the 20% height level on the RBC curve

Normal Range = 39–47 fL

When used in combination with the MCV, the RDW and RDW-SD are suggested to be useful in the early detection of iron deficiency anemia and in distinguishing between iron deficiency anemia (increased RDW) and beta (β)-thalassemia minor (normal RDW).^{1,2}

Platelet Analysis

The **platelet histogram** displays native platelet size. Particles with volumes ranging from 2 to 20 fL are counted. The raw data is then fitted to a curve from 0 to 70 fL (Fig. 32-2), from which the reported platelet count is derived. This curve-fitting technology ensures accurate platelet counting even with the presence of very large platelets or very small RBCs. The platelet counting range on the DxH 900 Series analyzers is extended to $0\text{--}3,000 \times 10^9/\text{L}$. The mean platelet volume (MPV) and platelet distribution width (PDW) are additional parameters describing platelet size and anisocytosis. The MPV is the platelet equivalent of the MCV and is inversely proportional to the platelet count. The PDW

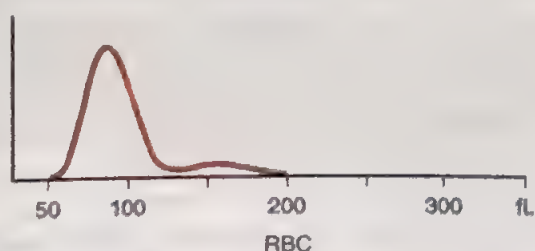


FIGURE 32-1 Coulter RBC histogram (Courtesy of Beckman Coulter Inc., Brea, CA.)

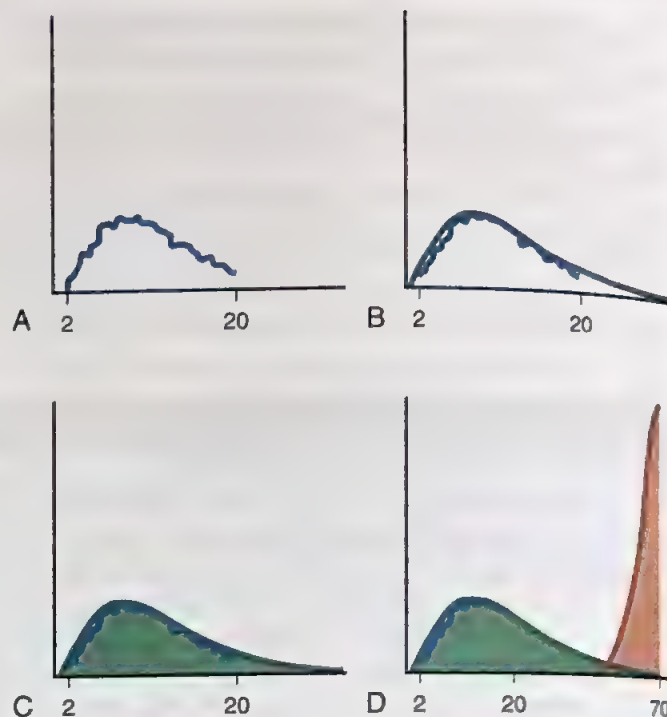


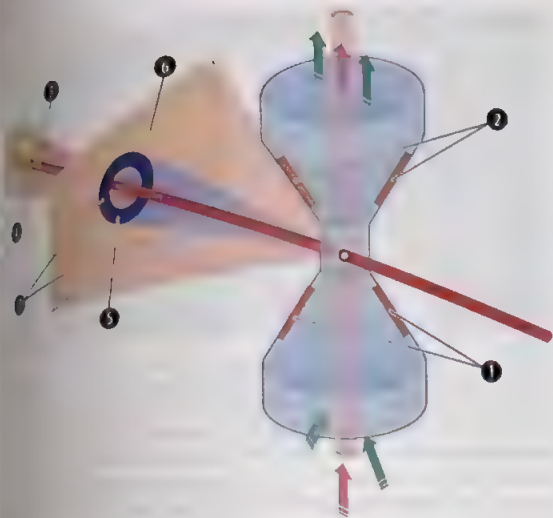
FIGURE 32-2 Coulter platelet count determination. **A.** The platelet histogram has a lower limit of 2 fL and an upper limit of 20 fL. **B.** Using the least squares regression formula, the formula defines a log normal curve. The log normal curve is plotted from 0 to 70 fL to include giant platelets. **C.** Integral calculus is used to find the area under the curve. The area under the curve is calibrated to a reference platelet count. **D.** RBC pulses between 0 and 70 fL are excluded from the PLT count by the electronic curve. The extrapolated area under the PLT curve includes even giant platelets. (Courtesy of Beckman Coulter Inc., Brea, CA.)

measures uniformity of platelet size and is equivalent to the RDW. In combination with the platelet histogram, the MPV and PDW provide valuable information on possible platelets. Measurement of platelet volume has been determined to yield diagnostic and prognostic value, with increased platelet volume characteristic of disorders marked by external platelet destruction and decreased values reflective of production disorders originating in the bone marrow.³ However, studies have shown that the MPV and PDW have limited predictive sensitivity and specificity in the diagnosis of bone marrow failure for patients presenting with thrombocytopenia.^{4,5}

Leukocyte Analysis

The VCS 360 measurement system has been significantly enhanced on the DxH Series system by utilization of a novel multitransducer module, which measures additional angles of light scatter, compared with previous analyzers that relied upon a singular measurement angle (Fig. 32-3). Leukocyte analysis is performed in three dimensions, and individual cells are depicted as points on a scatterplot reflecting cell volume, conductivity, and light-scattering characteristics (Fig. 32-4). NRBCs and reticulocytes are also counted in the VCS 360 module.

Results from each of the approximately 8,000 analyzed WBC (or 32,000 red cells in the reticulocyte analysis) are assigned X, Y, Z coordinates in a three-dimensional array based respectively on their rotated light scatter (RLS), volume, and opacity. Clusters of cells are identified as lymphocytes (blue), monocytes (green), neutrophils (purple), eosinophils (orange), basophils (white).



area plots illustrating peak height. Improved Unicel® technology algorithms provide optimal separation of raw values to define unusual population shifts, overlaps, and irregular or deficient separation to decrease incidence of the WBC* flag, spurious elevation of the WBC count due to interfering substances, and detection of nucleated red blood cells.^{6,7}

Reticulocyte Analysis

Automatic reticulocyte analysis combines the established methodology of the new methylene blue procedure with the standardized analysis and greater precision of flow cytometry utilizing the VCS technology. In doing so, it provides high-quality results without the need for fluorescent dyes and argon ion laser systems. It also allows the user to provide improved reproducible data to clinicians. Within the analyzer, a small segment of the blood sample is incubated in a heated chamber with a special new methylene blue solution precipitating any residual RNA within the erythrocytes. A portion of the stained blood sample is then transferred to a second chamber together with a hypotonic clearing solution that will remove erythrocyte hemoglobin but preserve the stained RNA within the cell. Almost immediately, the sample is processed through the VCS flow cell for analysis by the same three independent energy probes used for differential analysis. Contour gating is of particular benefit in reticulocyte analysis because the cell population under study is a gradual continuum of increasing maturity. The nonlinear separation techniques and multiresolution analysis algorithm allow the DxH series analyzers to provide improved precision and accuracy of results, particularly in the presence of abnormal RBC types (Fig. 32-5). Advanced parameters, such

FIGURE 32-3 Light scatter on the DxH 900/DxH 690T. Upper and lower electrodes measure volume and conductivity using low- (DC) and high-frequency (RF) currents (1, 2). Light scatter is measured in multiple channels to provide information regarding internal complexity: 0° Axial light loss (3), 5.1° Low Angle Light Scatter (4), 10°-20° Lower Median Angle Light Scatter (5), 20°-42° Upper Median Angle Light Scatter (6), and the sum of the Upper and Lower Median Angle Light Scatter (7).



FIGURE 32-4 The five-part differential is determined using three energy probes (Volume, Conductivity, and Laser Light Scatter) and plotting individual cells in three dimensions. The VCS cube displays the WBC populations separate and distinct populations differentiated by color: Neutrophils (blue), Lymphocytes (green), Monocytes (orange), Eosinophils (red), Basophils (purple), and non-WBC (red). (Courtesy of Beckman Coulter Inc., Brea, CA.)

and NRBCs (red) based on their signature position within the scatterplot. Separate scatterplots comparing cell volume and conductivity properties are generated to quantify basophils. Data models can be manipulated to view in 2-D (volume and conductivity), 3-D (density, light scatter, and opacity), or as surface



FIGURE 32-5 Using the same energy probes as the WBC differential and the supravital stain new methylene blue, reticulocytes are plotted in a three-dimensional scatterplot. The colors of the scatterplot indicate red blood cells (red), platelets (green), and reticulocytes (purple). Additional reticulocyte parameters are derived from a variety of information provided by the VCS technology. (Courtesy of Beckman Coulter Inc., Brea, CA.)

as immature reticulocyte fraction (IRF) and mean reticulocyte volume (MRV), provide additional information regarding reticulocyte quantity and quality of the reticulocyte population. These advanced parameters can be useful in the diagnosis and monitoring of anemic patient populations.⁸

Nucleated Red Blood Cell Detection

A nucleated red blood cell (NRBC) count is provided with every differential. The proprietary NRBC counting method utilizing VCS 360 technology identifies particles in a specific location within the VCS 3-D cube seen in Figure 32-5. This NRBC “signature position,” in combination with the interfering cells seen at the lower discriminator of the WBC histogram, indicates that NRBCs are present. A corrected WBC count is generated if there is significant interference in the sample as seen in the WBC histogram (Fig. 32-6).

Abnormal Flags

Contour discriminators examine areas between different cell populations. Abnormalities are identified by specific **region flags** (R-flags). The flagging system is enhanced by providing specific alphanumeric codes and three types of messages including suspect, definitive, and condition messages. Common abnormalities associated with each flagging message appear in Table 32-2. Suspect messages flag abnormal cell populations or distributions such as certain RBC abnormalities, platelet clumping, variant lymphocytes, immature granulocytes, and blasts. These messages appear in the cell classification

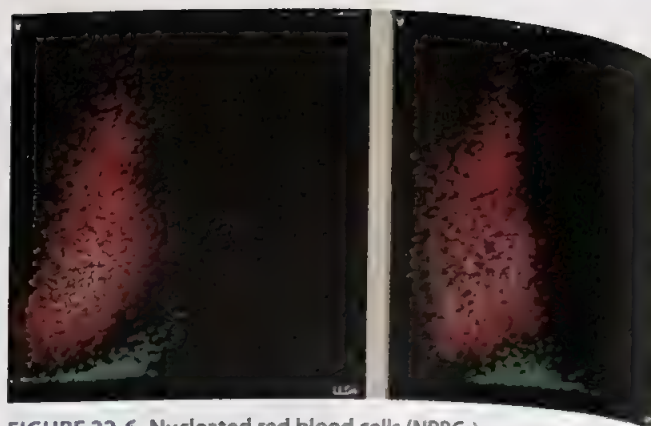


FIGURE 32-6 Nucleated red blood cells (NRBCs) are calculated when the analyzer identifies cells present in a signature position for NRBCs in the VCS scatterplot and detects interference at the lower discriminator of the WBC histogram. If there are enough cells present to significantly interfere with the WBC count, the count is corrected for the presence of the interference. Both the corrected WBC count and the uncorrected WBC count are available. (Display image LH700 series, Courtesy of Beckman Coulter Inc., Brea, CA.)

window on the sample analysis display and suggest confirmation by slide review. Condition messages indicate whether WBC, RBC, or platelet populations are abnormal or normal.

Body Fluid Analysis

Accurate counting of cells in body fluids is required to inform clinicians of the possibility of inflammatory, infective, or neoplastic processes. Cell counts (total nucleated cells and red blood cells) on body fluids are available on DxH Series

TABLE 32-2 Summary of Coulter DxH Series Analyzer Flagging Messages

Population	Suspect Messages	Condition Messages	Definitive Messages
WBC	<ul style="list-style-type: none"> Immature granulocytes Left shift Variant lymphocytes Blasts NRBC Review slide 	<ul style="list-style-type: none"> Normal WBC population Abnormal WBC population Abnormal DIFF pattern Cellular interference 	<ul style="list-style-type: none"> Leukocytosis Leukopenia Neutrophilia Neutropenia Monocytosis Lymphocytosis Lymphopenia Eosinophilia Thrombocytopenia
RBC	<ul style="list-style-type: none"> Dimorphic RBC population RBC fragments RBC agglutination Sickled cells Abnormal hemoglobin 	<ul style="list-style-type: none"> Normal RBC population Abnormal RBC population Abnormal retic pattern 	<ul style="list-style-type: none"> Anemia Microcytosis Macrocytosis Anisocytosis Poikilocytosis Hypochromia Pancytopenia Erythrocytosis Reticulocytosis
Platelets	<ul style="list-style-type: none"> Giant platelets Platelet clumping 	<ul style="list-style-type: none"> Normal platelet population 	<ul style="list-style-type: none"> Thrombocytopenia Thrombocytosis Large platelets Small platelets

hematology analyzers. Three categories of body fluids can be analyzed efficiently and accurately: CSF, serous fluids (pleural, peritoneal, pericardial), and synovial fluids that have been treated with hyaluronidase.^{9,10}

Additional Parameters

In addition to flagging abnormal cells, DxH hematology analyzers can generate other informative data categorized as research population data and subcategorization of cell population data for various WBC, RBC/reticulocyte, and body fluid cell populations (Fig. 32-7). Although for research and investigational purposes only at this stage, a number of recently published reports have suggested some of these parameters are valuable in detecting early stages of myelodysplastic syndrome, investigating abnormal lymphocytes and immature granulocytes, identifying sepsis, viral infection, and the presence of intracellular parasites.^{6,11-15}

CRITICAL THINKING QUESTION

32-1 What are the RBC indices measured with instrument analysis?

See answers to all Critical Thinking Questions at the back of this book.

Specimen Evaluation by Light Scattering and Cytochemical Analysis: ADVIA® Hematology Systems, Siemens Healthcare Diagnostics

The ADVIA is a flow cytometry-based system that uses light scatter, differential white blood cell lysis, and myeloperoxidase and oxazine 750 staining to provide a complete blood cell count (CBC), a WBC differential, and reticulocyte counts from whole blood. Select models can also provide cell counts and differential for CSF fluids. The system uses **Unifuidics** technology and consists of an analytical module, along with an optional auto-sampler and a data manager (Fig. 32-8). The analyzer uses two separate and independent flow cytometers to count and identify blood cells. Once a sample is aspirated, it is delivered into a ceramic shear valve, where it is divided into separate aliquots for analysis (Fig. 32-9). Reagents and aliquots are subsequently drawn into respective reaction chambers: hemoglobin reaction chamber for the colorimetric measurement of hemoglobin concentration, a combined RBC and platelet reaction chamber, two channels (the peroxidase and lobularity/nuclear density chambers) for WBC counts and differentials and a reticulocyte reaction chamber. Cells are counted and analyzed as they pass through a flow

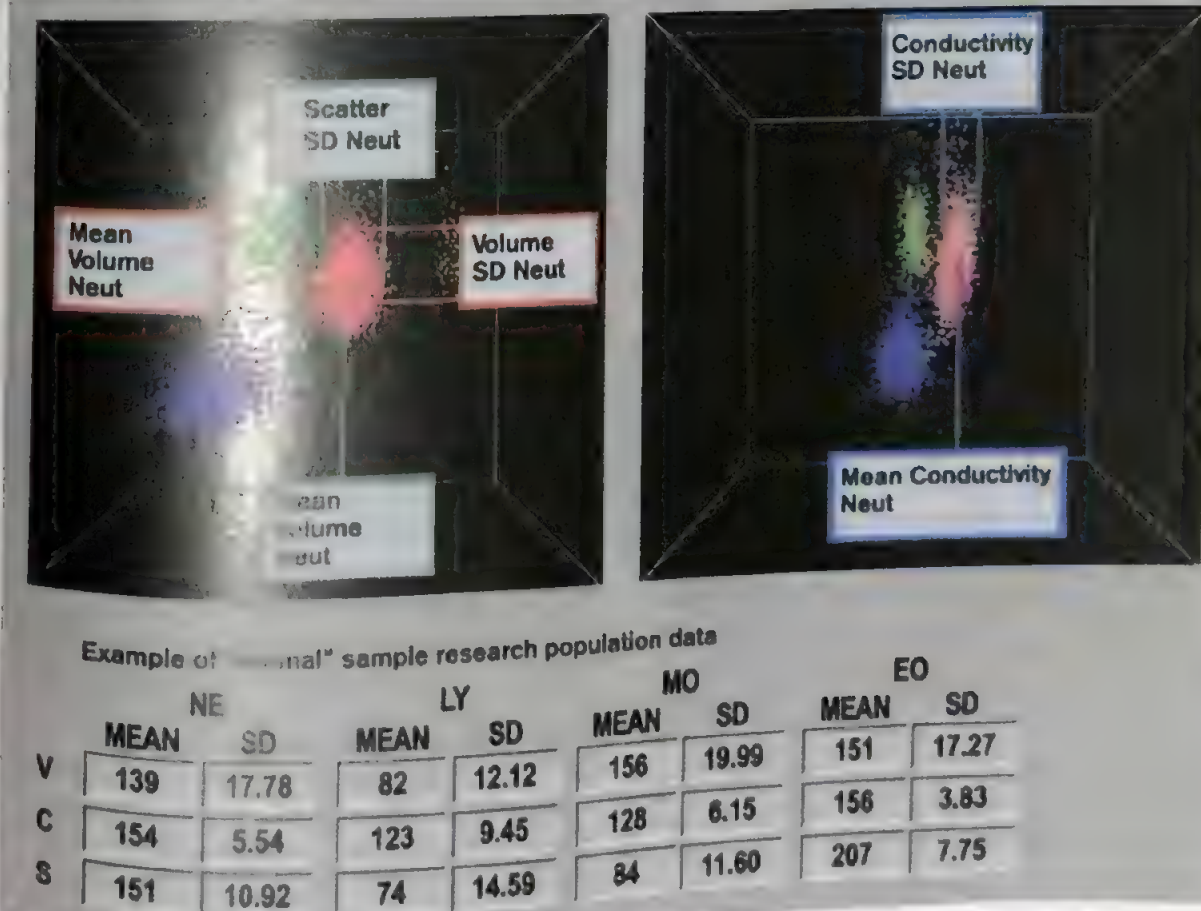


FIGURE 32-7 For each of the neutrophil, lymphocyte, monocyte, eosinophil, reticulocyte, and nonreticulocyte (RBCs in the reticulocyte count) populations, a mean and standard deviation is displayed for the volume, conductivity, and light scatter measurements. Those values appear as Research Population Data (RPD) on the WBC and Retic display tabs. (Courtesy of Beckman Coulter Inc., Brea, CA.)



FIGURE 32-8 ADVIA 2120 System. (Courtesy of Siemens Healthcare Diagnostics, Tarrytown, NY.)



FIGURE 32-9 The Unifluidics technology of the ADVIA 2120 Hematology System. This circuit is composed of acrylic blocks that provide pathways for fluid transport, airflow, valves, and reaction chambers. (Courtesy of Siemens Healthcare Diagnostics, Tarrytown, NY.)

cell. Cytograms derived from various cell analysis channels are shown in Figure 32-10.

The ADVIA 120/2120 accommodate the sample throughput necessary for high-volume laboratories. The newest generation, the ADVIA 2120i system, offers full automation and an integrated slide maker. The system provides a reticulocyte count, a six-part differential, and optional CSF analysis. The ADVIA 560/560L, built for midvolume laboratories, but offers a five-part differential and lacks the capability to provide a reticulocyte count or perform CSF analysis. The ADVIA 360 provides a three-part differential and is best suited for low-volume environments.

Red Cell Analysis

Red blood cells and platelets are identified based on their light-scattering properties. A buffered reagent isovolumetrically fixes and spheres red cells, while light scattered at high (5 to 15 degrees) and low (2 to 3 degrees) angles concurrently

measures cell volume and cell optical density. An RBC scatter cytogram is a graphic representation of these measurements, where the high-angle light scatter is plotted on the x-axis and the low-angle light scatter on the y-axis (see Fig. 32-10A). The RBC cytogram map resolves volumes between 1 and 180 fL and refractive index values between 1.33 and 1.44. Unique red blood cells indices are directly measured by means of mathematical theory termed Lorenz-Mie theory, which provides an equation to convert light scattering measurements into cell volume and hemoglobin concentration.¹⁶

The ADVIA 120/2120 system directly measures the volumes and hemoglobin concentrations of individual RBCs and therefore allows the determination of chromasia and erythrocyte size on a cell-by-cell basis. This information is presented in an RBC (V/HC) cytogram (Fig. 32-11). The hemoglobin concentration (HC) is plotted along the x-axis and cell volume along the y-axis. RBCs are further characterized as hypochromic, normochromic, and hyperchromic based on hemoglobin markers set at 28 g/dL and 41 g/dL. RBCs with a hemoglobin concentration less than 28 g/dL are characterized as hypochromic, and cells with a hemoglobin concentration greater than 41 g/dL are characterized as hyperchromic. Values within the range of 28 g/dL and 41 g/dL are normochromic. Distribution of cells based on hemoglobin concentration is represented on the HC histogram. Samples with normal values exhibit a bell-shaped curve between 28 g/dL and 41 g/dL. The mean hemoglobin concentration defines the corpuscular hemoglobin concentration mean (CHCM) for red cells. This measurement is analogous and alternative to the classic mean corpuscular hemoglobin concentration (MCHC) derived from the hemoglobin concentration and the hematocrit. In the case of a decreased MCHC with increased numbers of hypochromic red cells, the histogram will be skewed to the left, indicating red cells with hemoglobin concentration of less than 28 g/dL. Conversely, in the case of an increased MCHC, the histogram will be skewed to the right, indicating the presence of hyperchromic red cells with hemoglobin concentration greater than 41 g/dL.

Cell markers for RBC volume are used to identify different cell sizes, such as microcytic, normocytic, and macrocytic cells. The RBC cell volume markers are set at 60 and 120 fL. RBCs with a volume less than the 60 fL are characterized as microcytic, and red cells with a volume greater than 120 fL are characterized as macrocytic. Values within the range of 60 fL and 120 fL are normocytic.

The RBC volume histogram provides information for the MCV and RDW measurements. The histogram has a range of 0 to 200 fL. Samples with normal values exhibit a bell-shaped curve between 60 and 120 fL. The MCV is the mean of the RBC volume histogram. In the case of a decreased MCV with increased numbers of microcytic red cells, the histogram will be skewed to the left, indicating red cells with volumes less than 60 fL. Conversely, in the case of an increased MCV, the histogram will be skewed to the right, indicating the presence of macrocytic red cells with volumes greater than 120 fL. The RDW is a measurement of anisocytosis and is calculated from the CV of the red cell population. The RDW will be flagged when the value exceeds 16%.

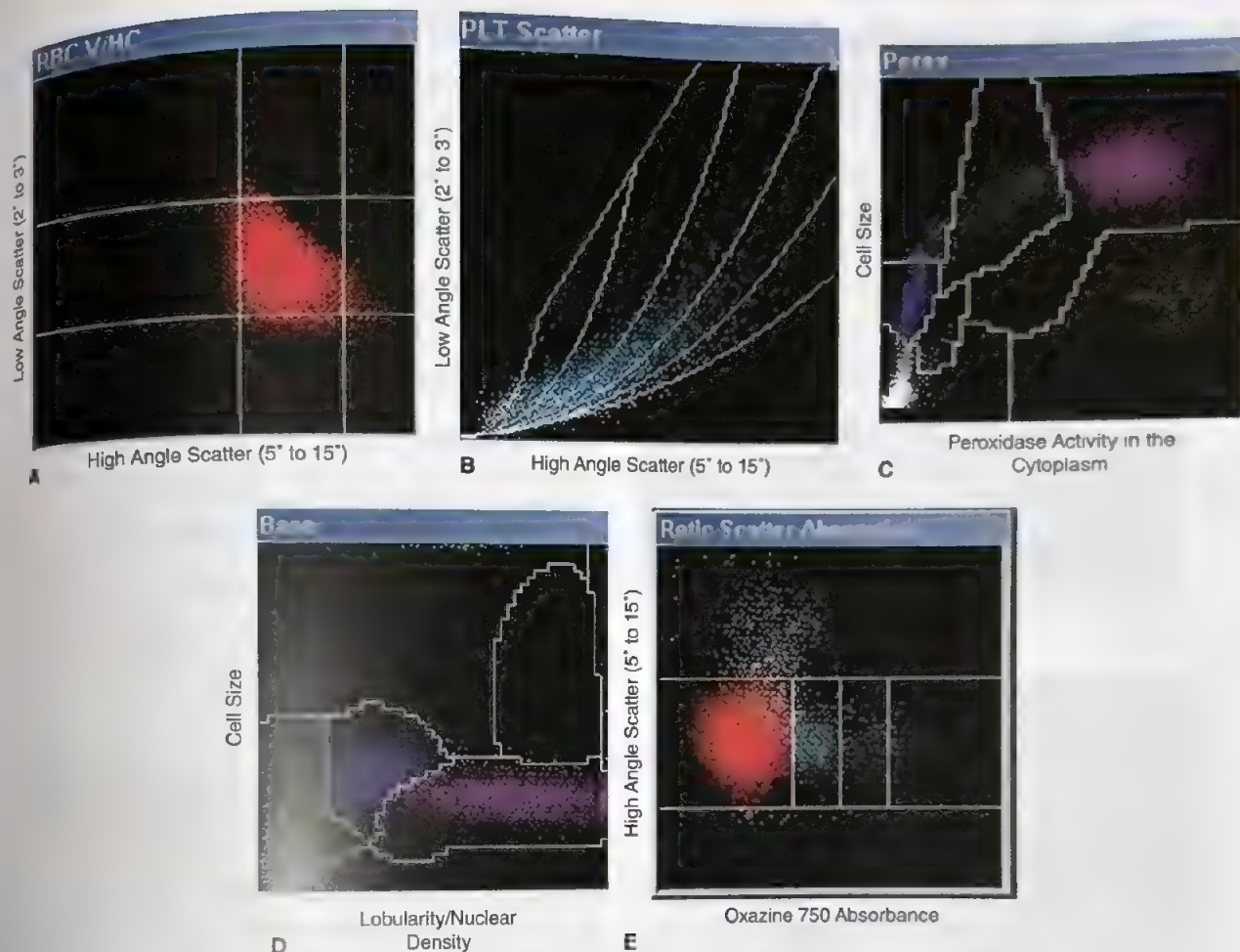


FIGURE 32-10 Cytograms derived from the various channels of the ADVIA 2120 Hematology System. **A.** RBC scatter cytogram. **B.** Platelet scatter cytogram. **C.** Peroxidase cytogram. **D.** Lobularity/Nuclear density (basophil channel) cytogram. **E.** Reticulocyte cytogram. (Courtesy of Siemens Healthcare Diagnostics, Tarrytown, NY.)

The availability of red cell parameters such as percentages of microcytic, macrocytic, hypochromic, and hyperchromic cells may provide valuable information for assessing red blood cell disorders. Clinical applications for some of these parameters have already been described. For example, most of the RBCs in a patient with iron deficiency will be both hypochromic and microcytic, whereas microcytosis will be more pronounced than hypochromasia in patients with a thalassemic trait.⁸ Such indices are also useful to characterize and distinguish distinct hemoglobinopathy and thalassemia diagnoses.¹⁷⁻¹⁹

Platelet Analysis

Platelet enumeration takes place in the RBC reaction chamber. The respective aliquot of sample passes through the flow cell, where low-angle and high-angle light scatter are detected and electronically amplified. In the case of platelets, low-angle light scatter is amplified 30 times, and high-angle light scatter is amplified 12 times. Using the Mie theory²⁰ of light scattering for homogenous spheres, the low-angle and high-gain (amplified signals) scatter is converted into

cell volume and high-angle and high-gain scatter is converted to a refractive index. The ADVIA 120/2120 produces a platelet scatter cytogram that plots the data respective to cell volume and refractive index, and a platelet map grid that lists ranges for cell volume (0 to 30 fL) and refractive index (1.3500 to 1.4000) (see Fig. 32-10B). The reportable platelet count comprises both normal-sized platelets and large platelets with volumes up to 60 fL. This two-dimensional analysis allows discriminating platelets from other nonplatelet particles, and microcytes, RBC fragments and RBC ghost cells are excluded from the platelet count.

Leukocyte Analysis

Two separate methods are used on the ADVIA 120/2120 system to analyze white blood cells. A total WBC count is measured from two reaction chambers, the peroxidase chamber and the lobularity/nuclear density chamber. In the peroxidase chamber, WBCs are fixed with formaldehyde and stained with peroxidase reagents in the peroxidase reaction chamber. The chamber is heated to a relatively high temperature, which lyses platelets and RBCs and causes the WBCs

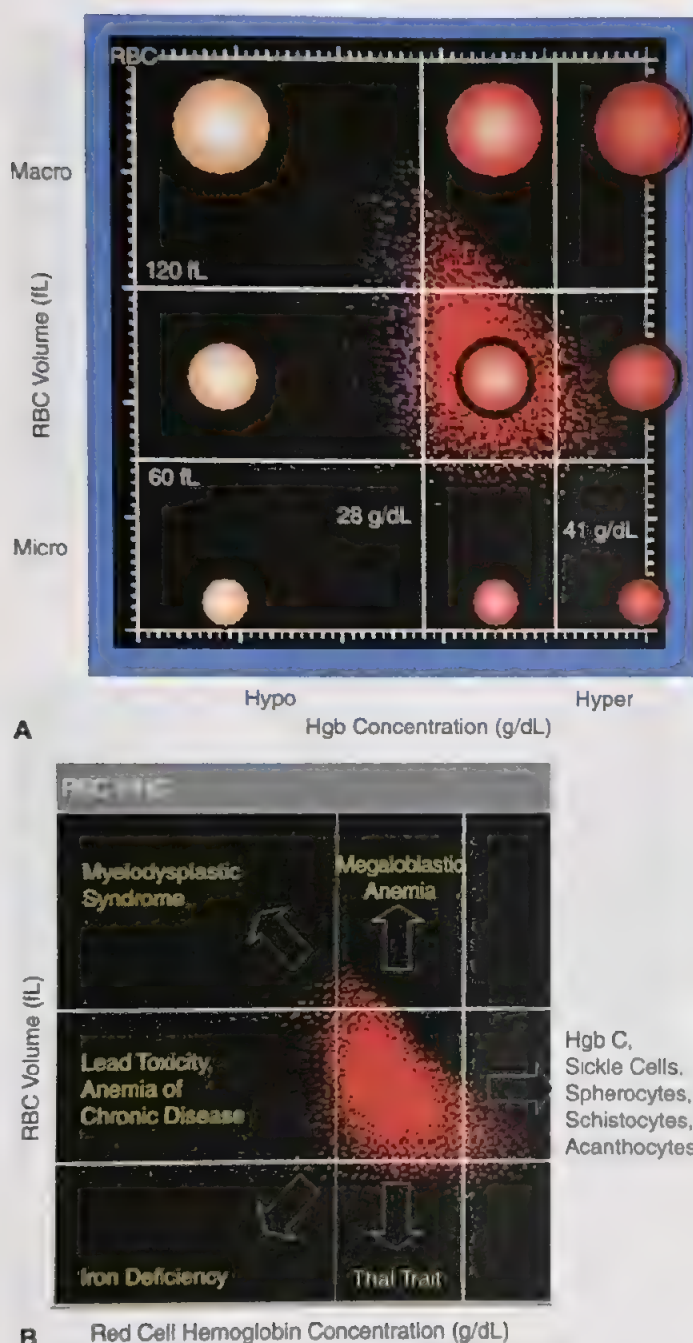


FIGURE 32-11 Siemens ADVIA 120/2120 red blood cell cytograms. **A.** RBC cytogram and the position of hypochromic, normochromic, hyperchromic, microcytic, normocytic, and macrocytic cells. **B.** An increase of certain clusters of RBCs is consistent in various disease states. (Harris, N, et al: *The ADVIA 2120 Hematology System: A flow cytometry-based analysis of blood and body fluids in the routine hematology laboratory.*) (Courtesy of Siemens Healthcare Diagnostics, Tarrytown, NY.)

to be fixed. Narrow forward-angle light scatter and tungsten light optics are used to measure WBC size and peroxidase activity, respectively. Myeloperoxidase is a granulocyte enzyme marker that is present to varying degrees in neutrophils, eosinophils, and monocytes but absent from basophils, lymphocytes, and blasts. The data are displayed on a PEROX cytogram with light absorption depicted on the x-axis and forward scatter on the y-axis. Cluster analysis is applied to identify distinct populations of cell clusters that can further

be categorized by position, area, and density on the cytogram (see Fig. 32-10C).

In the lobularity/nuclear density reaction chamber, a whole blood aliquot is exposed to an acid buffer that selectively lyses the cytoplasm of all cells except basophils. The sample passes through the flow cell, where low-angle light scatter and high-angle light scatter are measured. Because basophils are resistant to lysis, they appear larger than the bare nuclei of other leukocytes, scatter more light, and appear higher on the vertical axis of the scattergram. The size of the cell or nucleus and nuclear configuration (nuclear shape and cell density) are analyzed. The BASO cytogram consists of 100 channels on each axis. High-angle light scatter or nuclear configuration is plotted on the x-axis, and low-angle light scatter or cell size is plotted on the y-axis. As a result, distinct clusters of cells are formed and analyzed respective of the cells' position, area, and density on the cytogram (see Fig. 32-10D). A primary WBC count and a basophil count are derived from this channel.

The WBC differential report generated by the ADVIA 120/2120 system not only indicates the relative percentages and absolute values for neutrophils, lymphocytes, monocytes, eosinophils, and basophils, but also provides additional interpretative data to signal the presence of sample abnormalities. The differential report also includes the percentage of large unstained cells (LUCs). Increased numbers of LUCs may reflect the presence of atypical lymphocytes or blasts.

Reticulocyte Analysis

Reticulocyte analysis is performed using oxazine 750, a nucleic acid dye that selectively stains reticulocytes and distinguishes them from mature red cells (see Fig. 32-10E). The measurements include low angle light scatter (2 to 3 degrees), high-angle light scatter (8 to 15 degrees), and absorption analysis. The two light scatter measurements are proportional to cell size and hemoglobin concentration, respectively, and the light absorption is proportional to the content of RNA in stained reticulocytes. Mature red cells and immature red cells (reticulocytes) are analyzed at the same time providing additional information on the interrelationship of these two populations. The results of these three parameters are plotted on a reticulocyte volume histogram, on a hemoglobin concentration histogram, and on the reticulocyte hemoglobin content histogram. The reticulocyte hemoglobin content (CHr) has proven useful in the diagnosis and management of anemia^{21,22} in adult and pediatric patients.²³

Nucleated Red Blood Cell Detection

The ADVIA 2120i identifies the presence of NRBC in the peroxidase and basophil measurement channels. The number of NRBC present is estimated as the difference between the number of nucleated cells quantitated in the neutrophil/eosinophil area in the Basophil channel and the sum of the neutrophils and eosinophils present in the Peroxidase channel.²⁴ The sensitivity and specificity of this method correlates well with manual microscopy detection.²⁵ However, because it is an indirect measurement, it carries greater measurement error

compared with direct measurement techniques, possibly from interfering substances.^{7,24}

Abnormal Flags

Morphology flags on the ADVIA 120/2120 Analyzer are designed based on flagging algorithms for the purpose of alerting laboratory personnel to sample abnormality. When a flag is triggered, the operator should perform the prescribed corrective action before results are reported. Flagging is divided into three entities: suspect morphology, flag, and triggering criteria. Table 32-3 gives examples of morphology flagging available on the ADVIA 120/2120 analyzer.

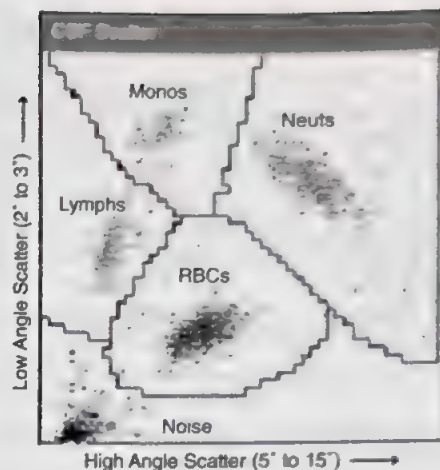
Cerebrospinal Fluid Analysis

Enumeration of RBCs and WBC in CSF samples is performed using direct cytometry on the ADVIA 120/2120 system. An aliquot of CSF sample is pretreated with the CSF reagent that fixes and spheres the cells. The cells are differentiated and enumerated based on three optical measurements: high-angle scatter, low-angle scatter, and absorbance. The data are displayed on the CSF cytograms (Fig. 32-12) and reportable results include WBC and RBC counts and percentages and absolute values for mononuclear cells, polymorphonuclear cells, neutrophils, lymphocytes, and monocytes.

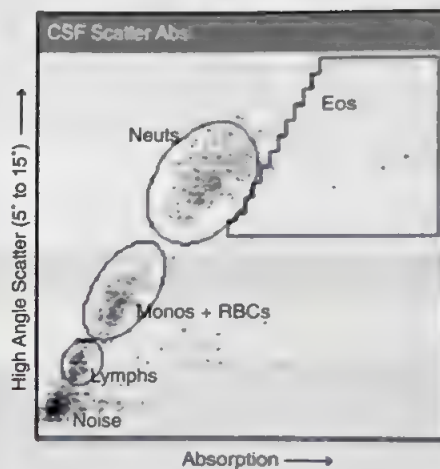
TABLE 32-3 Examples of Morphology Flagging by the ADVIA 120/2120 Hematology Analyzer

Suspect Morphology	Flag	Triggering Criteria
Anisocytosis	ANISO	RDW \geq 16%
Atypical lymphocytes	ATYP	% LUC \geq 4.5 and % LUC \geq (% BLASTS plus 1.5)
Blasts	BLAST	% BLASTS 1.5% to 5% and % LUC \geq 4.5%, or BLASTS \geq 5% WBCB and (%Baso + %Baso Susp + %BAso Sat) \geq 10%
Hemoglobin concentration variation	HCVAR	HDW \geq 3.4 g/dL
Hyperchromia	HYPER	% HYPER \geq 4%
Hypochromia	HYPO	% HYPO \geq 4%
Immature granulocytes	IG	(% NEUT + % EOS) - % PMN \geq 5%
NRBC	NRBC	+ if NRBC are enumerated
Large platelets	LPLT	% LPLT $>$ 10% PLT
Macrocytosis	MACRO	% MACRO \geq 2.5%
Microcytosis	MICRO	% MICRO \geq 2.5%
Platelet clumps	PLT-CLM	Clumps count $>$ 150

RDW = red cell distribution width, LUC = large unstained cells, HDW = hemoglobin distribution width, NEUT = neutrophils, EOS = eosinophils, PMN = polymorphonuclear cell, NRBC = nucleated red blood cell, LPLT = large platelet.
Source: ADVIA 2120 Operators Manual, Siemens Healthcare Diagnostics, Tarrytown, NY.



A



B

FIGURE 32-12 ADVIA 2120 CSF assay cytograms. **A.** CSF scatter cytogram and the position of red cells (RBCs), lymphocytes (Lymphs), monocytes (Monos), neutrophils (Neuts). **B.** Scatter/absorption cytogram and relevant position of various cells. (Courtesy of Siemens Healthcare Diagnostics, Tarrytown, NY.)

CRITICAL THINKING QUESTION

32-2 What is forward angle light-scatter used to measure?

Specimen Evaluation With Hydrodynamic Focusing, RF/DC Technology, and Fluorescent Flow Cytometry: The Sysmex XN and XN-L Series Hematology Analyzers

Sysmex America, Inc. produces a comprehensive series of multiparameter hematology analyzers (XN and XN-L Series) that use electronic resistance, or direct current (DC), with hydrodynamic focusing for counting RBCs and platelets. Fluorescent flow cytometry and light scatter are used for the WBC count, PLT count, WBC differential, and NRBC

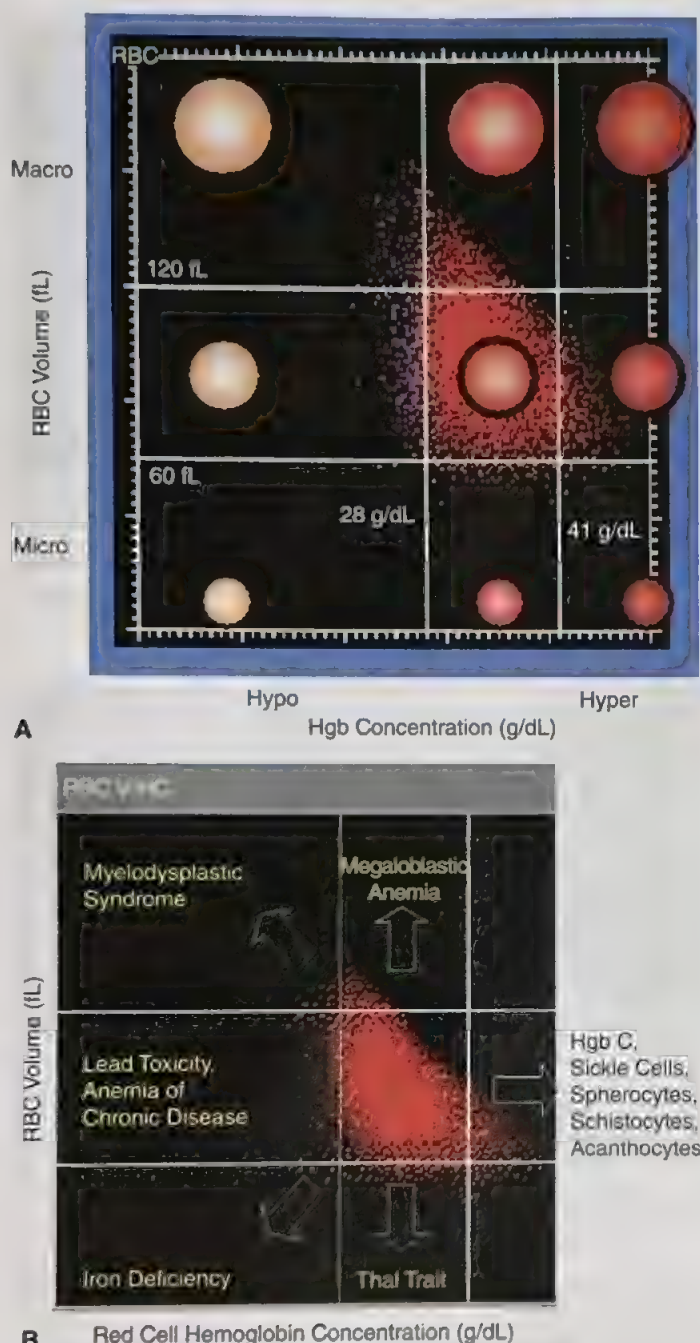


FIGURE 32-11 Siemens ADVIA 120/2120 red blood cell cytograms. **A.** RBC cytogram and the position of hypochromic, normochromic, hyperchromic, microcytic, normocytic, and macrocytic cells. **B.** An increase of certain clusters of RBCs is consistent in various disease states. (Harris, N, et al: The ADVIA 2120 Hematology System: A flow cytometry-based analysis of blood and body fluids in the routine hematology laboratory.) (Courtesy of Siemens Healthcare Diagnostics, Tarrytown, NY.)

to be fixed. Narrow forward-angle light scatter and tungsten light optics are used to measure WBC size and peroxidase activity, respectively. Myeloperoxidase is a granulocyte enzyme marker that is present to varying degrees in neutrophils, eosinophils, and monocytes but absent from basophils, lymphocytes, and blasts. The data are displayed on a PEROX cytogram with light absorption depicted on the x-axis and forward scatter on the y-axis. Cluster analysis is applied to identify distinct populations of cell clusters that can further

be categorized by position, area, and density on the cytogram (see Fig. 32-10C).

In the lobularity/nuclear density reaction chamber, a whole blood aliquot is exposed to an acid buffer that selectively lyses the cytoplasm of all cells except basophils. The sample passes through the flow cell, where low-angle light scatter and high-angle light scatter are measured. Because basophils are resistant to lysis, they appear larger than the bare nuclei of other leukocytes, scatter more light, and appear higher on the vertical axis of the scattergram. The size of the cell or nucleus and nuclear configuration (nuclear shape and cell density) are analyzed. The BASO cytogram consists of 100 channels on each axis. High-angle light scatter or nuclear configuration is plotted on the x-axis, and low-angle light scatter or cell size is plotted on the y-axis. As a result, distinct clusters of cells are formed and analyzed respective of the cells' position, area, and density on the cytogram (see Fig. 32-10D). A primary WBC count and a basophil count are derived from this channel.

The WBC differential report generated by the ADVIA 120/2120 system not only indicates the relative percentages and absolute values for neutrophils, lymphocytes, monocytes, eosinophils, and basophils, but also provides additional interpretative data to signal the presence of sample abnormalities. The differential report also includes the percentage of large unstained cells (LUCs). Increased numbers of LUCs may reflect the presence of atypical lymphocytes or blasts.

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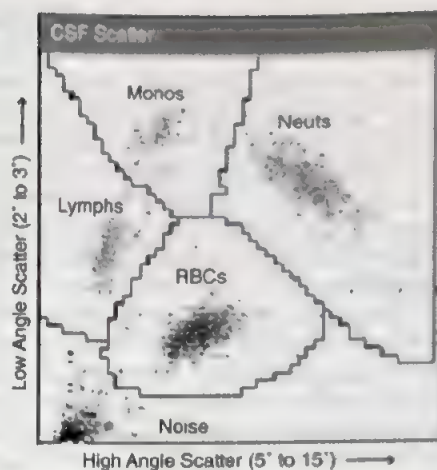
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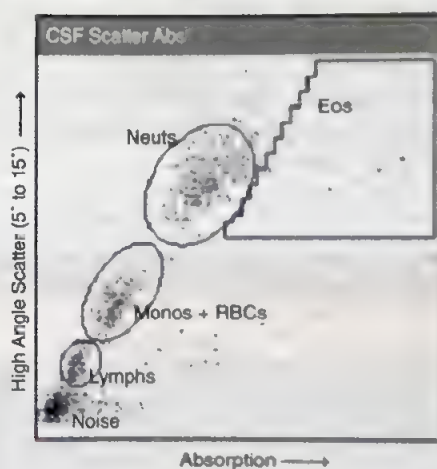
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RDW = red cell distribution width; LUC = large unstained cells; HDW = hemoglobin distribution width; NEUT = neutrophils; EOS = eosinophils; PMN = polymorphonuclear cell; NRBC = nucleated red blood cell; LPLT = large platelet
Source: ADVIA 2120 Operators Manual, Siemens Healthcare Diagnostics, Tarrytown, NY



A



B

FIGURE 32-12 ADVIA 2120 CSF assay cytograms. **A.** CSF scatter cytogram and the position of red cells (RBCs), lymphocytes (Lymphs), monocytes (Monos), neutrophils (Neuts). **B.** Scatter/absorption cytogram and relevant position of various cells. (Courtesy of Siemens Healthcare Diagnostics, Tarrytown, NY.)

CRITICAL THINKING QUESTION

32-2 What is forward angle light-scatter used to measure?

Specimen Evaluation With Hydrodynamic Focusing, RF/DC Technology, and Fluorescent Flow Cytometry: The Sysmex XN and XN-L Series Hematology Analyzers

Sysmex America, Inc. produces a comprehensive series of multiparameter hematology analyzers (XN and XN-L Series) that use electronic resistance, or direct current (DC), with hydrodynamic focusing for counting RBCs and platelets. Fluorescent flow cytometry and light scatter are used for the WBC count, PLT count, WBC differential, and NRBC

and reticulocyte counts. The XN-Series analyzers perform hematology analysis by fluorescent flow cytometry using a semiconductor laser and cell-specific stains. Hemoglobin is measured using sodium lauryl sulfate (SLS-hemoglobin method). These instruments generate multiple scattergrams, depending on the tests requested, as well as red cell and platelet histograms. Information reported is customizable by institution and may include WBC differential, fluorescent platelet, NRBC, and reticulocyte scattergrams, as well as RBC and platelet histogram data.

In 2012, Sysmex released the XN-Series followed by the XN-L Series in 2016. The XN-Series offers compact solutions for small to medium facilities as well as scalable configurations for large laboratory facilities. The XN-Series provides a total of 31 reportable analytic parameters including the routine CBC and six-part WBC differential, RBC count, fluorescent platelet, and comprehensive reticulocyte analysis. The XN-L Series provides full CBC and six-part differential technology for small laboratories. The XN-Series can process up to 100 samples per hour while the XN-L Series can process up to 60 samples per hour. See Table 32-4 for specifications of the XN and XN-L Series analyzers.

Red Cell Analysis

The XN and XN-L Series analyzers count and size red blood cells using focused hydrodynamic flow impedance. This method reduces coincidence counting and variant pulses due to nonaxial flow and recirculation of cells into the sensing zone. Red cells and platelets pass through the aperture of the detector surrounded by sheath fluid using laminar flow. Sheath fluid, which is under slight pressure, surrounds the column of cells, aligning the particles in single file and guiding them through the aperture. When the particles pass through the transducer aperture, they interrupt an electronic current and generate a pulse, thereby determining the size of the cell. Cells are swept away by the sheath fluid and recirculation is prevented. RBCs are represented by histograms with floating discriminators (Fig. 32-13). These floating discriminators reduce the effect of interfering substances and adjust to individual patient cell populations.

Hemoglobin analysis is performed using a sodium lauryl sulfate (SLS) reagent, which binds with the hemoglobin and forms SLS-Hb. An advantage to the SLS-hemoglobin method is that it does not contain cyanide. Additionally, common interfering substances such as lipemia, high WBC counts, and

TABLE 32-4 Specifications of the XN and XN-L Series Analyzers

Specifications	XN-Series (XN-10, XN-20)	XN-L Series (XN-330, XN-430, XN-530, XN-350, XN-450, XN-550)
Sample volume	Sampler Mode – 88 μ L Manual (Closed Cap) Mode – 88 μ L Manual (Open Cap) Mode – 88 μ L Dilution Mode – 70 μ L Body Fluid Mode – 88 μ L	Sampler Mode – 25 μ L Manual (Closed Cap) Mode – 25 μ L Manual (Open Cap) Mode – 25 μ L Dilution Mode – 70 μ L Body Fluid Mode – 70 μ L
Parameters	Whole Blood Mode: WBC, RBC, HGB, HCT, MCV, MCH, MCHC, PLT, NEUT%/#, LYMPH%/#, MONO%/#, EO%/#, BASO%/#, NRBC%/#, RDW-CV, RDW-SD, MPV, RET%/#, IRF, IG%/#, RET-He#, IPF Body Fluid Mode: WBC-BF, RBC-BF, MN%/#, PMN%/#, TC-BF#	Whole Blood Mode: WBC, RBC, HGB, HCT, MCV, MCH, MCHC, PLT, NEUT%/#, LYMPH%/#, MONO%/#, EO%/#, BASO%/#, RDW-CV, RDW-SD, MPV, RET%/#, IRF, IG%/#, RET-He# Body Fluid Mode: WBC-BF, RBC-BF, MN%/#, PMN%/#, TC-BF#
Reagents	SULFOLYSER™ (Lyse) CELLPACK™ DCL (Diluent) CELLPACK™ DST™ (Diluent)* CELLPACK™ DFL™ (Diluent) LYSERCELL™ WNR™ (Lyse) LYSERCELL™ WDF™ (Lyse) LYSERCELL™ WPC™ (Lyse)* FLUOROCELL™ WNR™ (Stain) FLUOROCELL™ WDF™ (Stain) FLUOROCELL™ RET (Stain) FLUOROCELL™ PLT (Stain) FLUOROCELL™ WPC™ (Stain)*	SULFOLYSER™ (Lyse) CELLPACK™ DCL (Diluent) CELLPACK™ DFL™ (Diluent) LYSERCELL™ WDF™ (Lyse) FLUOROCELL™ WDF™ (Stain) FLUOROCELL™ RET (Stain)
Principles	Performs Hematology analyses using hydrodynamic focusing (DC Detection), flow cytometry (using a semiconductor laser), and SLS-hemoglobin method.	Performs Hematology analyses according to the Hydro Dynamic Focusing (DC Detection), flow cytometry method (using a semiconductor laser), and SLS hemoglobin method
Dimensions (H × W × D) (inches)	33.7 × 25.4 × 29.7*	20.08 × 17.72 × 18.11*

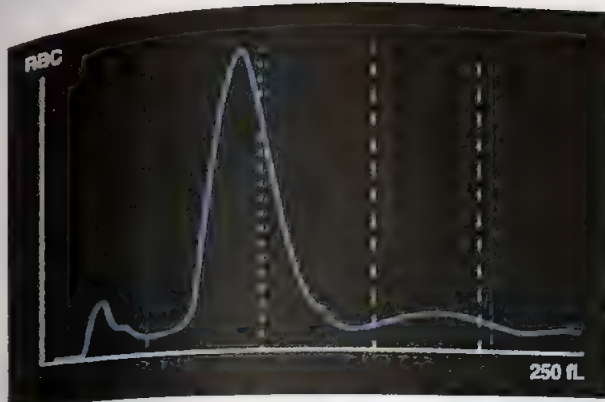


FIGURE 32-13 Normal RBC histogram on a Sysmex X-Series analyzer. (Sysmex Instrument Technology, Mundelein, IL, with permission.)

abnormal proteins present fewer problems with this method. Hematocrit is directly measured through cumulative pulse height detection and is determined by summing the volume of each cell as it passes through the orifice during the RBC count. This produces a hematocrit result that closely matches the reference method, or spun hematocrit, result.

The red cell distribution width (RDW) is reported as both the RDW-SD (standard deviation) and RDW-CV (coefficient of variation). The RDW-SD is an actual measurement (in femtoliters) of the width of the RBC histogram. This measurement is made at a point 20% above the baseline. Because the RDW-SD is an actual measurement, it is not affected by the average size of the RBCs (MCV). The RDW-CV is a mathematically derived parameter calculated using the formula $RDW-CV = (1\text{ SD}/MCV) \times 100$, where 1 SD reflects the size variation of the RBCs around the mean. Because the 1 SD is divided by the MCV, the RDW-CV measurement is dependent on the average size of the RBCs or the MCV (Fig. 32-14).

Platelet Analysis

Platelets are also analyzed using hydrodynamic focused flow impedance and represented by histograms with floating discriminators (Fig. 32-15). The XN-Series can utilize fluorescent flow cytometry for confirmatory platelet analysis. In cases where giant platelets or RBC fragments interfere with the impedance platelet count (PLT-I), the fluorescent platelet (PLT-F) channel available on the XN-Series analyzers provide an additional platelet determination, utilizing fluorescent flow cytometry and a platelet-specific reagent dye. Giant platelets can be identified by fluorescent flow cytometry and are easily included in the platelet population. RBC fragments do not interfere with the platelet count because their size and fluorescence intensity places them away from the platelet population.

Leukocyte Analysis

The total WBC count and WBC differential are determined by fluorescent flow cytometry. Each cell is analyzed by three different dispersion angles: forward-scattered light, side-scattered light, and side fluorescent light. The XN-Series analyzers have two dedicated channels (WDF and WNR) for leukocyte differential analysis; the XN-L Series

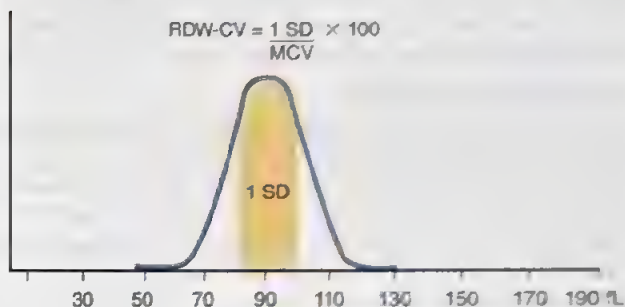
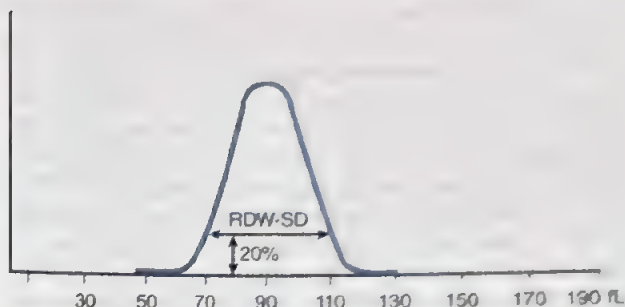


FIGURE 32-14 Sysmex determination of RDW-SD and RDW-CV. (Walters, J. et al.: RDW-SD and RDW-CV: Their relationship to RBC distribution curves and anisocytosis. *Sysmex J Int* 3(1): 40, 1993.)

PLT-I

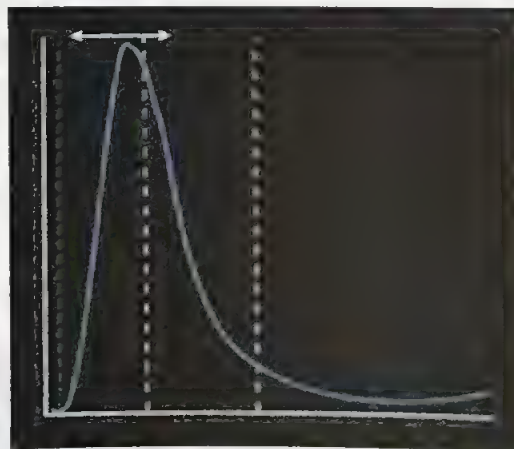


FIGURE 32-15 Normal platelet histogram on a Sysmex X-Series analyzer. (Sysmex Instrument Technology, Mundelein, IL, with permission.)

analyzers use only the WDF channel. The WNR channel uses a combination of a cell-specific lyse, a fluorescent stain, and fluorescent flow cytometry to provide a nucleated red blood cell count, WBC count, and differential analysis for basophils. Lysercell™ WNR™ reagent lyses and dissolves red cells and platelets and makes holes in the leukocyte membrane. The polymethine dye contained in the Fluorocell™ WNR™ reagent then enters the white cells and stains nucleic acids and cytoplasmic organelles. The cells are then analyzed using a semiconductor laser.

The WDF Channel on XN and XN-L Series analyzers differentiates and counts neutrophils, lymphocytes, monocytes, eosinophils, and immature granulocytes. It also has advanced flagging algorithms to detect abnormal cells such as immature white blood cells and atypical lymphocytes. Surfactants in Lysercell™ WDF™ cause the hemolysis and dissolution of red blood cells and platelets and penetrates the cell membranes of white blood cells. Then the fluorescent dye in Fluorocell™ WDF™ enters the cells and stains the nucleic acids and cell organelles. The intensity of fluorescence varies among different types of white blood cells, depending on the type and amount of nucleic acids present. It is possible to differentiate and count various cells and flag abnormal cells through the differences in side-scattered light and fluorescence intensity (Fig. 32-16).

Reticulocyte Analysis

Reticulocytes are enumerated by fluorescent flow cytometry using a semiconductor laser and a nucleic acid fluorescent dye. Red cells are stained with fluorescent dye and illuminated by the laser. This produces a specific wavelength intensity or forward fluorescence directly proportional to the quantity of RNA in each cell. By analyzing both forward scattered light and side fluorescence information, the reticulocyte count and reticulocyte maturity indices are obtained. The red cell population is separated into mature erythrocytes and reticulocytes (Fig. 32-17). Also determined in this channel is the RET-He, or reticulocyte hemoglobin equivalent. The RET-He is a clinically useful parameter to diagnose iron deficient cell production and monitor the response to corrective therapy.⁸

Nucleated Red Blood Cell Detection

Nucleated red blood cells are counted and distinguished from WBC in the WNR channel. After cells have been preferentially lysed and stained using Fluorocell™ WNR™ reagent, the analyzer measures nucleic acid content using side fluorescence and size using forward scatter (Fig. 32-18). The distinct differences in size and nucleic acid content differentiate nucleated red blood cells from white blood cells and thus enable a separate count to be provided for each.⁷

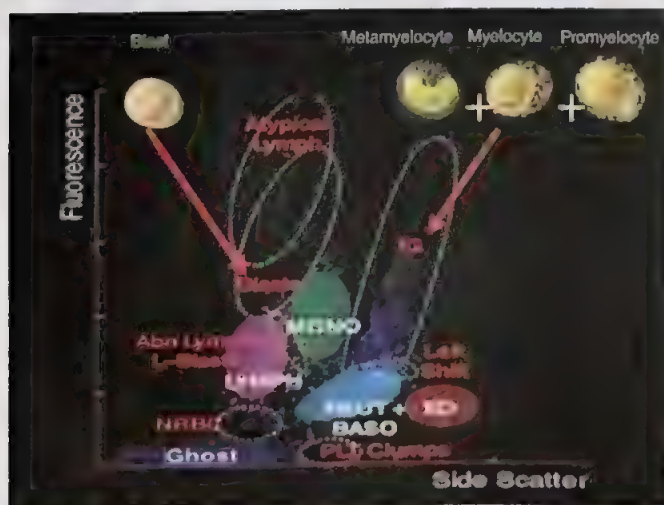


FIGURE 32-16 Location of normal and abnormal cell populations on a WBC differential scattergram from a Sysmex X-series analyzer. (Sysmex XE Series Technology, Mundelein, IL, with permission.)



FIGURE 32-17 Sysmex reticulocyte scattergram showing differentiation of immature reticulocyte, mature RBC, and optical platelet populations. (Sysmex XE Series Technology, Mundelein, IL, with permission.)



FIGURE 32-18 Normal NRBC scattergram from a Sysmex X-series analyzer. (Sysmex XE Series Technology, Mundelein, IL, with permission.)

Abnormal Flags

Sample abnormalities are indicated by flags or error messages that appear on the display screen and on the result printout. These flags can be customized based upon a laboratory's predefined limits or are generated by instrument software algorithms to indicate results that require further review and investigation. Using preset criteria, numerical data, particle size distributions, and scattergrams, analyzed samples are judged as POSITIVE or NEGATIVE. With this information, the XN and XN-L Series analyzers generate flags/messages that are referred to as IP (Interpretative Program) Messages. The sensitivity of many of the flags can be adjusted to meet the laboratory's needs. Table 32-5 provides examples of flagging messages generated by Sysmex analyzers.

TABLE 32-5 Examples of Flagging Messages Generated by Sysmex Analyzers

Parameter Class	Abnormal or Suspect	Flag Message	Generated From	Operator Definable?	Instrument Generated?
WBC	Abnormal	WBC Abn Scattergram	WDF and WNR* Scattergrams		Yes
		NRBC Present*	High Nucleated RBC* count (%)	Yes	
		Neutropenia	Low Neutrophil count (#/%)	Yes	
		Neutrophilia	High Neutrophil count (#/%)	Yes	
		Lymphopenia	Low Lymphocyte count (#/%)	Yes	
		Lymphocytosis	High Lymphocyte count (#/%)	Yes	
		Monocytosis	High Monocyte count (#/%)	Yes	
		Eosinophilia	High Eosinophil count (#/%)	Yes	
		Basophilia	High Basophil count (#/%)	Yes	
		Leukocytopenia	Low Leukocyte count (#)	Yes	
		Leukocytosis	High Leukocyte count (#)	Yes	
		IG Present	High Immature Granulocyte count (#/%)	Yes	
	Suspect	Blasts/Abn Lympho?	WDF Scattergram		Yes
		Blasts?*	WDF and WPC* Scattergrams		Yes
		Abn Lympho?*	WDF and WPC* Scattergrams		Yes
		Left Shift?	WDF Scattergram		Yes
		NRBC?*	WDF Scattergram		
		Atypical Lympho?	WDF Scattergram		Yes
RBC	Abnormal	RBC Abn Distribution	Impedance Histogram		Yes
		Dimorphic Population	Impedance Histogram		Yes
		RET Abn Scattergram*	RET* Scattergram		Yes
		Reticulocytosis*	High Reticulocyte count (#/%)	Yes	
		Anisocytosis	Elevated RDW-SD (#) or RDW-CV (%)	Yes	
		Microcytosis	Decreased MCV (#)	Yes	
		Macrocytosis	Increased MCV (#)	Yes	
		Hypochromia	Decreased MCHC (#)	Yes	
		Anemia	Decreased HGB (#)	Yes	
		Erythrocytosis	Increased RBC (#)	Yes	
	Suspect	RBC Agglutination?	Impedance Histogram		Yes
		Turbidity/HGB Interf?	Hemoglobin related parameters		Yes
		Iron Deficiency?	Impedance Histogram and Hemoglobin related parameters		Yes
		HGB Defect?	Impedance Histogram		Yes
		Fragments?	Impedance Histogram and RET* Scattergram		Yes
PLT	Abnormal	PLT Abn Distribution	Impedance Histogram		Yes
		PLT Abn Scattergram*	PLT-F* Scattergram		Yes
		Thrombocytopenia	Decreased Platelet count (#)	Yes	
		Thrombocytosis	Increased Platelet count (#)	Yes	
		PLT Clumps?	WNR*, WDF, and PLT-F* Scattergrams		Yes
	Suspect				

WBC = white blood cell; NRBC = nucleated red blood cell; IG = immature granulocyte; Baso = basophil; Diff = differential; Gran = granulocyte; Lympho = lymphocyte; RBC = red blood cell; Abn = abnormal; RET = reticulocyte; RDW-SD = red cell distribution width-standard deviation; RDW-CV = red cell distribution width-coefficient of variation; MCV = mean corpuscular volume; MCHC = mean corpuscular hemoglobin concentration; Hgb = hemoglobin; MCH = mean corpuscular hemoglobin; PLT = platelet.

* = dependent on XN or XN-L Series analyzer types.

Source: Adapted from Sysmex XN-Series Instructions for Use and XN-L Series Troubleshooting Guides, Sysmex America Inc., Lincolnshire, IL, with permission.

Body Fluid Analysis

CSF, serous fluid, and synovial fluid analysis can be performed on the XN and XN-L Series analyzers. Specimens are analyzed in the body fluid mode to provide enumeration of the WBCs and RBCs. Fluorescent flow cytometry using side-scattered light and fluorescent light are used to determine the WBC count, and DC detection is used for the RBC count. Bilevel body fluid QC specifically designed for use on the XN and XN-L Series analyzers is available. WBC counts $\geq 0.050 \times 10^3/\mu\text{L}$ and RBC counts $\geq 0.01 \times 10^6/\mu\text{L}$ can be reported. Lower counts require verification by an alternate method.

Specimen Evaluation by Multi-Angle Polarized Scatter Separation (MAPSS™) Technology: Abbott Alinity h-Series

The Alinity h-series is the latest high-volume hematology system designed by Abbott to meet the needs of the core laboratory. Along with the CELL-DYN systems, Abbott maintains a complete range of hematology solutions to serve different laboratory settings from small facilities to high volume, highly automated reference laboratories. Abbott hematology analyzers use a variety of technologies that include optical light scatter, fluorescence, impedance, and absorbance to perform the Complete Blood Count (CBC) and differential (Diff) analysis and measure reticulocytes. The Alinity h-series is an integrated hematology solution that includes the Alinity hq analyzer module and the Alinity hs slide maker and stainer module and is available in various configurations. The Alinity hq is a fully optical analyzer that uses spectrophotometry and optical and fluorescence flow cytometry to generate a CBC-Diff with 29 reportable parameters. Table 32-6 summarizes the primary analysis methods used by this technology.

The primary method of detection for most parameters on the Alinity hq is optical light scatter analysis. A beam of laser light is passed through a diluted blood specimen stream that is projected into the flow cell by hydrodynamic focusing (Fig. 32-19). As each cell passes through, the focused light is scattered in various directions and detected by photodetectors which convert the signal into an electric

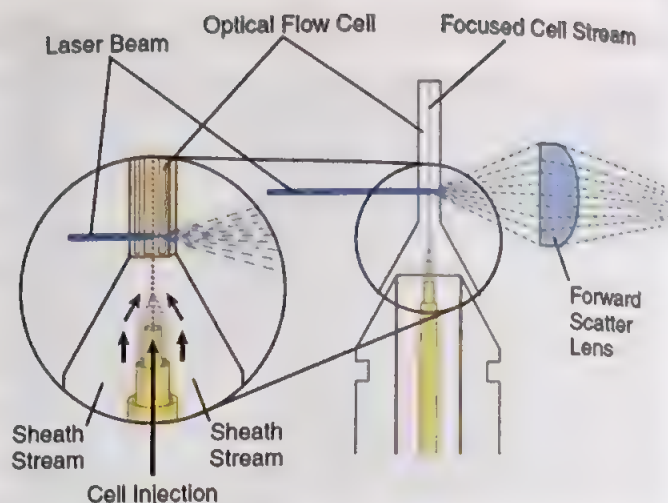


FIGURE 32-19 Hydrodynamic focusing used by the Abbott Alinity hq. This is the process where a suspension of cells is injected into a rapidly moving "sheath" of diluent. This has the effect of ensuring that the cells are focused in a path through the center of the laser light beam.

pulse. The electronic signals are transmitted to a computer for storage and analysis. The signals provide information about cellular characteristics such as size, internal complexity, nuclear lobularity/segmentation, and granularity, which are used to identify the cells. Cells with similar light scatter properties form a cluster in the scatterplot and can be separated from other cell clusters using advanced software algorithms. The utilization of these different angles of light scatter is known as **Multi-Angle Polarized Scatter Separation (MAPSS)**.

MAPSS is a propriety process that uses the detection of scattered laser light measured at different angles, in combination with different filters, to separate cells in multiple dimensions. This permits the counting of the WBCs, derivation of a differential, separation of PLTs and RBCs, and the detection of different types of abnormal blood elements without the use of additional stains or dyes. MAPSS technology was introduced and utilized on previously described hematology analyzers including the CELL-DYN Ruby and CELL-DYN Sapphire. The Alinity hq uses an Advanced MAPSS technology to provide a six-part differential including immature granulocytes (IGs), improved RBC and PLT analysis, and additional reticulocyte parameters.

The differences between MAPSS and Advanced MAPSS are derived from additional light scatter detectors and an updated algorithm that analyzes more events. Advanced MAPSS technology utilizes a 488 nm blue diode laser and a total of eight light scatter detectors including Axial light loss (ALL), four intermediate angles of light scatter (IAS, IAS1, IAS2, and IAS3), polarized side scatter (PSS), depolarized side scatter (DSS), and one fluorescent channel (FL1). Table 32-7 and Figure 32-20 summarize the detectors, angles of light scatter, attributes, and application for each detector.

Red Cell Analysis

RBC analysis on the Alinity hq is optical and uses six scatter signals (ALL, PSS, and the four IAS signals) as shown

TABLE 32-6 Primary Analysis Methods Used by the Alinity hq

Application	Technology
White Blood Cells (WBC) + Diff	Optical and fluorescence flow cytometry
Red Blood Cells (RBC) / Platelets (PLT)	Optical and fluorescence flow cytometry
Hemoglobin (HGB)	Spectrophotometry
Reticulocytes	Optical and fluorescence flow cytometry

Source: Contributed by Abbott Hematology, used with permission.

TABLE 32-7 Angles of Light Scatter, Attributes, and the Applications of the Detectors on the Alinity hq

Detector	Angle	Attribute	Application
ALL	0	Cell size	All modes
IAS	3.2-7.6	Cell contents	All modes
IAS1	2.4-4.5	Hemoglobin	RBC, PLT, and reticulocyte
IAS2	4.7-5.7	Cell volume	RBC, PLT, and reticulocyte
IAS3	5.9-7.6	Cytoplasmic granulation	RBC, PLT, and reticulocyte
PSS	90 pol	Nuclear segmentation	All modes
DSS	90 depol	Eosinophil granules	CBC (eosinophils)
FL1	90 fluor	Fluorescence	CBC (nucleated cells), reticulocyte

Source: Contributed by Abbott Hematology, used with permission.

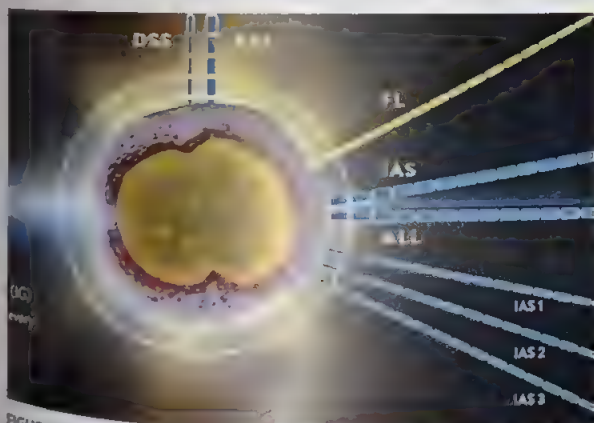


FIGURE 32-20 Example of the light scatter detectors used in the advanced MAPSS WBC analysis. (Courtesy of Abbott Laboratories, Abbott Park, IL.)

In Figure 32-21, Before analysis, RBC are isovolumetrically sphered to overcome orientation-dependent variation in light scatter and improve the accuracy of RBC volume determination.^{28,27} According to the Mie theory of light scattering for homogeneous spheres, monochromatic light scattered at two different forward angular intervals permits simultaneous determination of the volume and the HGB concentration of each cell.^{28,29} The Alinity hq accomplishes this using the IAS1 and IAS2 detectors.

Hemoglobin is measured on the Alinity h using absorption photometry. This method is based on the linear relationship between the amount of light absorbed in a well-mixed, stationary sample and the concentration of the analyte in a sample (as defined by Beer's Law). In the HGB analysis, RBCs are lysed using a cyanide-free reagent and the released

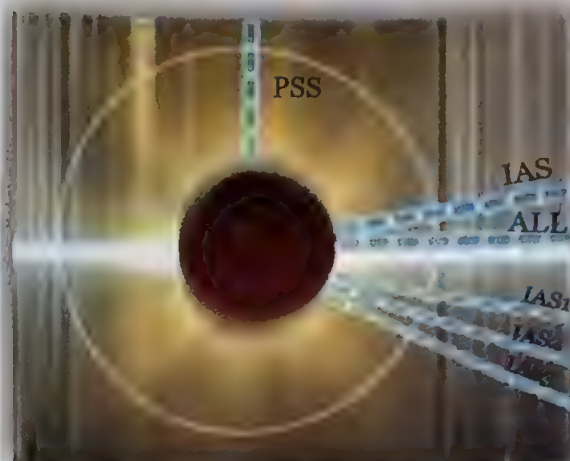


FIGURE 32-21 The different angles of light scatter used in the RBC analysis on the Alinity hq. The additional IAS angles improve separation between RBCs and PLTs and allow for the calculation of the cellular hemoglobin concentration. (Courtesy of Abbott Laboratories, Abbott Park, IL.)

hemoglobin is converted into a chromogen with an absorption peak of 540 nm (Fig. 32-22).³⁰ Multiple angles of scatter measurement are also used for cell-by-cell RBC volume and cellular hemoglobin concentration (CHC) analysis. The CHC × Volume scatterplot (Fig. 32-23) shows the optical RBC measurements graphed as a scatterplot with gridlines at 60 and 120 fL for volume and 28 g/dL and 41 g/dL for cellular hemoglobin concentration. This scatterplot can be used to

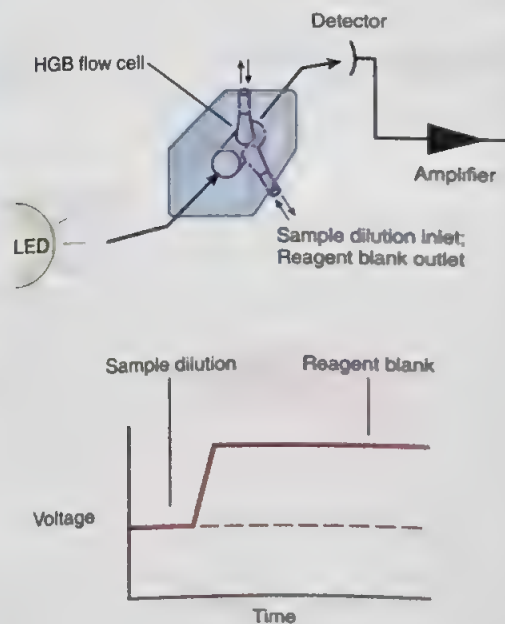


FIGURE 32-22 In the hemoglobin flow cell, the sample is illuminated by a 540 nm light-emitting diode (LED). A photodetector measures the amount of light that passes through the sample. (Courtesy of Abbott Laboratories, Abbott Park, IL.)

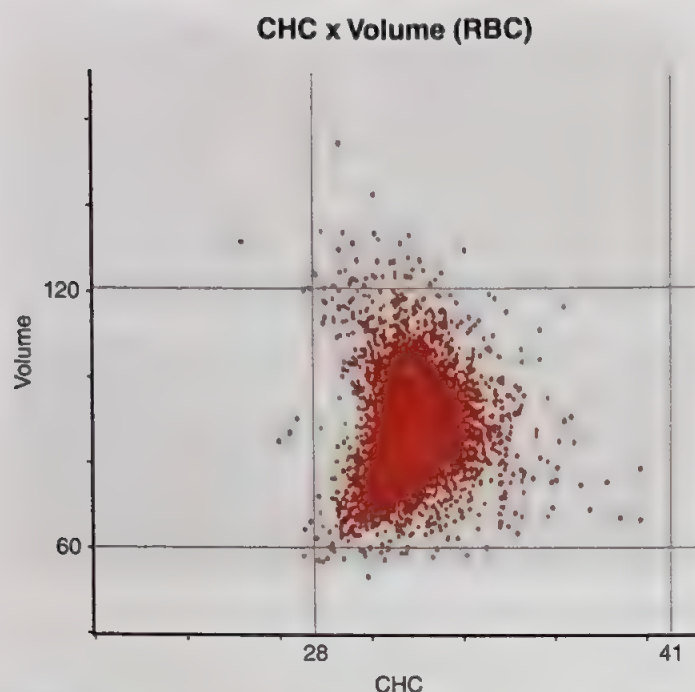


FIGURE 32-23 The RBC cellular hemoglobin concentration (x-axis) vs. RBC size (y-axis) is depicted here. The entire population of RBC is shown, and deviation from normal in terms of microcytosis, macrocytosis, hypochromia, and hyperchromia is visualized by four fixed thresholds that define hypochromic (<28 g/dL), hyperchromic (>41 g/dL), microcytic (<60 fL), and macrocytic (>120 fL) RBC. (Courtesy of Abbott Laboratories, Abbott Park, IL.)

indicate distributional abnormalities of both volume and cellular HGB concentration, as well as the presence of multiple RBC populations which may occur in myelodysplastic syndrome, response to treatment in iron deficiency, presence of cold agglutinins, or a recent RBC transfusion.³¹

Platelet Analysis

PLTs are measured optically using the ALL, IAS, IAS1, IAS2, IAS3, and PSS detectors, and measured concurrently with RBCs in a separate dilution from the one used for the evaluation of WBCs. Optical PLT counting methods allow for better separation between the cellular elements of blood because they collect signals that reflect the internal structure and complexity of the cells, in addition to their size. The Advanced MAPSS™ technology on the Alinity hq uses six detectors to distinguish PLTs from cells and particles of similar size such as microcytes, spherocytes, RBC fragments, and debris. Capturing the scattered light in multiple angles improves the differentiation of RBCs, PLTs, and other cellular events or particles by providing additional information about the cells and by creating unique optical signal signatures (Fig. 32-24A). This is particularly evident in cases where RBCs have low volume (microcytes, schistocytes, spherocytes) and the volume of these small/fragmented RBCs overlap with PLTs (Fig. 32-24B). In this respect, the multidimensional optical PLT counting method provides better differentiation than the impedance PLT counting method.^{24,32} The performance of the Alinity hq PLT count has been shown to be comparable to flow cytometry results. Bias at the $10.0 \times 10^9/L$ and $20.0 \times 10^9/L$ PLT transfusion thresholds was negligible compared with the international reference method.³³ These results suggest that Alinity hq delivers accurate PLT results in severely thrombocytopenic patients, supporting PLT transfusion decisions.

Leukocyte Analysis

During WBC analysis, the Alinity hq aspirates and dilutes whole blood, and the resulting cellular suspension is then exposed to lysing and membrane-permeable fluorescent dye

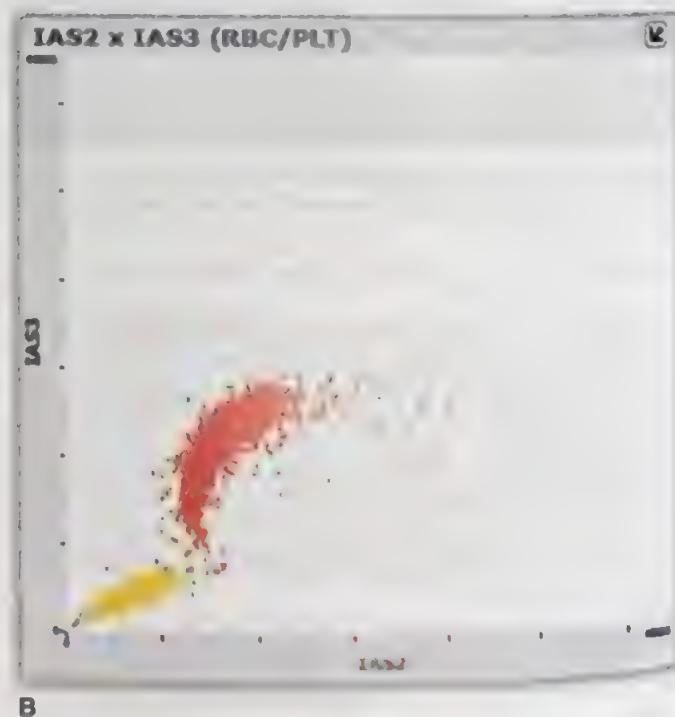
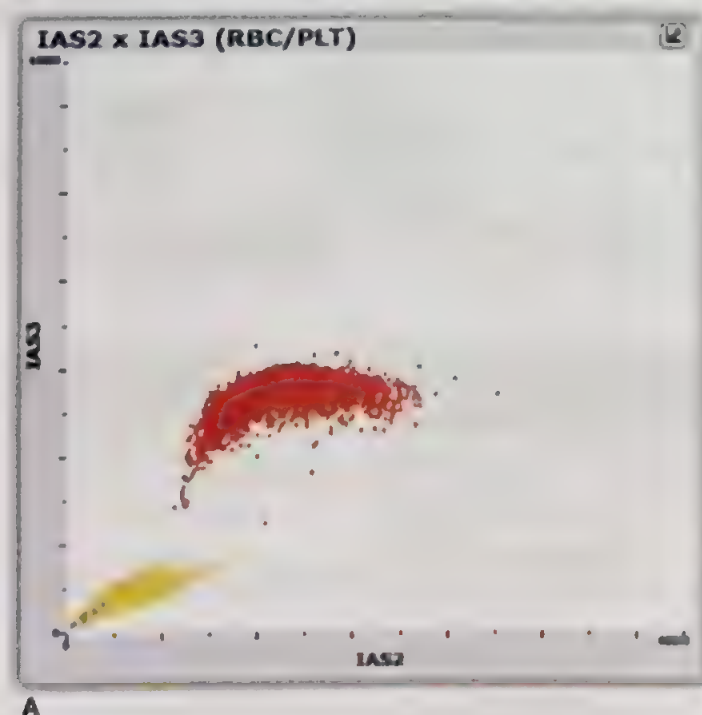


FIGURE 32-24 Separation of PLTs and RBCs in the IAS2 versus IAS3 scatterplot on the Alinity hq. **A.** Normal example showing clear differentiation between the RBCs (red) and the PLTs (yellow). **B.** Microcytic anemia showing a narrower gap (due to reduced size differences) but a well-defined separation between the RBC and PLT populations. (Courtesy of Abbott Laboratories, Abbott Park, IL.)

agents. This process destroys RBC (and NRBC) membranes while maintaining the WBCs in their near native state. The nuclei of the WBCs and any NRBCs are stained with fluorescent dye, which assists in cell classification. The cellular suspension then is injected into the optical flow cell, where the data from eight detectors are collected on up to 750,000 cellular events (Fig. 32-20). The eight detectors that are used to collect signals from each cell provide more information on cellular characteristics, thereby increasing the accuracy of cell identification and differentiation without the need for additional reagents. The addition of fluorescence flow cytometry captures the light emitted from internal cellular components that fell in front of the laser beam. This feature enhances the analysis, allowing for the quantification of IGs as a separate component of the differential, and ensuring that nonnucleated events such as lyse-resistant RBC and cellular debris do not interfere with the WBC count (Fig. 32-25).

After completion of raw data collection, the instrument's software algorithms perform a multidimensional analysis of the raw data, separating the cells into individual components of the WBC differential. Information from all the detectors is utilized, with measurements from certain detectors aiding in cell separation and classification. The IAS and PSS measurements are used to separate polymorphonuclear cells from mononuclear cells (lymphocytes and monocytes) based on complexity and lobularity (Fig. 32-26A). Eosinophils are differentiated based on the DSS signal, as the granules of eosinophils are more efficient than neutrophil granules in depolarizing light (Fig. 32-26B). Lymphocytes, monocytes, and basophils are separated using ALL (size) and IAS (complexity) (Fig. 32-26C) as part of the multidimensional analysis. NRBCs and nonfluorescent material are differentiated from WBCs based primarily

on their small size and fluorescence activity (Fig. 32-26D). IGs are enumerated separately in the six-part differential using a combination of their light-scatter principles and fluorescence. The separated and classified cells are displayed on the Alinity in colored scatterplots.

Reticulocyte Analysis

Reticulocyte measurement uses an aliquot taken from the RBC/PLT dilution and mixed with the reticulocyte reagent, which uses a fluorescent dye that binds to the RNA found in reticulocytes. The Alinity hq algorithm first separates RBCs from platelets and WBCs based on size and fluorescence properties and then differentiates mature RBCs with very low fluorescence from higher fluorescing reticulocytes using a dynamic threshold. A second dynamic threshold is used for separating the immature reticulocyte fraction (IRF), which have the highest fluorescent intensity, from more mature reticulocytes. The combination of optical flow cytometry with fluorescence to detect reticulocytes enables the determination of the Mean Cell Hemoglobin Content of the Reticulocyte (MCHr). MCHr (also referred to as Reticulocyte Cellular Hemoglobin Content [CHR] or Reticulocyte Hemoglobin equivalent [RET-He] by non-Abbott analyzers) is considered to be a measure of iron availability for erythropoiesis. The Alinity hq also provides a reportable % reticulated PLT count (%rP) parameter using a similar method to that used to separate IRF from mature reticulocytes. %rP is analogous to the immature platelet fraction or IPF on other non-Abbott analyzers.

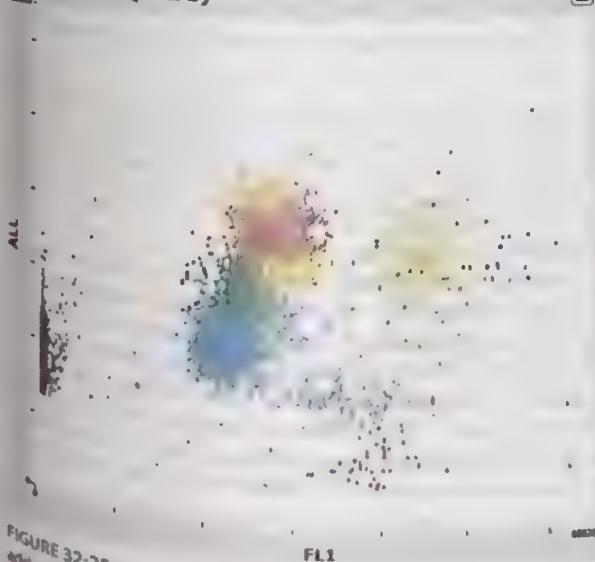
Nucleated Red Blood Cell Detection

NRBC detection and enumeration is included with every CBC processed on the Alinity hq. The membrane-permeable fluorescent nuclear dye facilitates the separation of nucleated cells (WBC and NRBC) from nonnucleated events, such as lyse-resistant RBC and cellular debris. Fluorescent emission from cell nuclei is measured with a detector in the FL1 channel (Fig. 32-27). This information allows NRBCs to be separated from WBCs and displayed as both an absolute number and number per 100 WBCs (NR/W).

Abnormal Flags

The Alinity hq offers a comprehensive flagging strategy that includes morphological flags, data invalidating flags, system messages, numerical result flags, and optional flags. Morphological flags are generated by the algorithm based on sample characteristics; they will suspect or invalidate a result, depending on the parameter and extent of the abnormal characteristics detected. Morphological flags on the Alinity hq are summarized in Table 32-8. Data invalidating flags and system messages typically indicate an issue with the system hardware or with the algorithm during sample processing and will invalidate potentially affected results. Numerical result flags are either limit set flags, for example, if a patient or QC result is outside of user defined limits, or calculation or display status flags where the system cannot calculate or display reliable results, for example, if the sample result is outside of the analytical measurement range (AMR). Optional flags are generated based on user-defined limits for numerical results and can be defined for most parameters.

FL1 x ALL (WBC)



FL1

FIGURE 32-25 Example scatterplot from the Alinity hq analyzer. The addition of fluorescent analysis allows for a valid WBC count in cases of lyse-resistant RBCs due to the cells lack of fluorescence (gray population of cells). (Courtesy of Abbott Laboratories, Abbott Park, IL.)

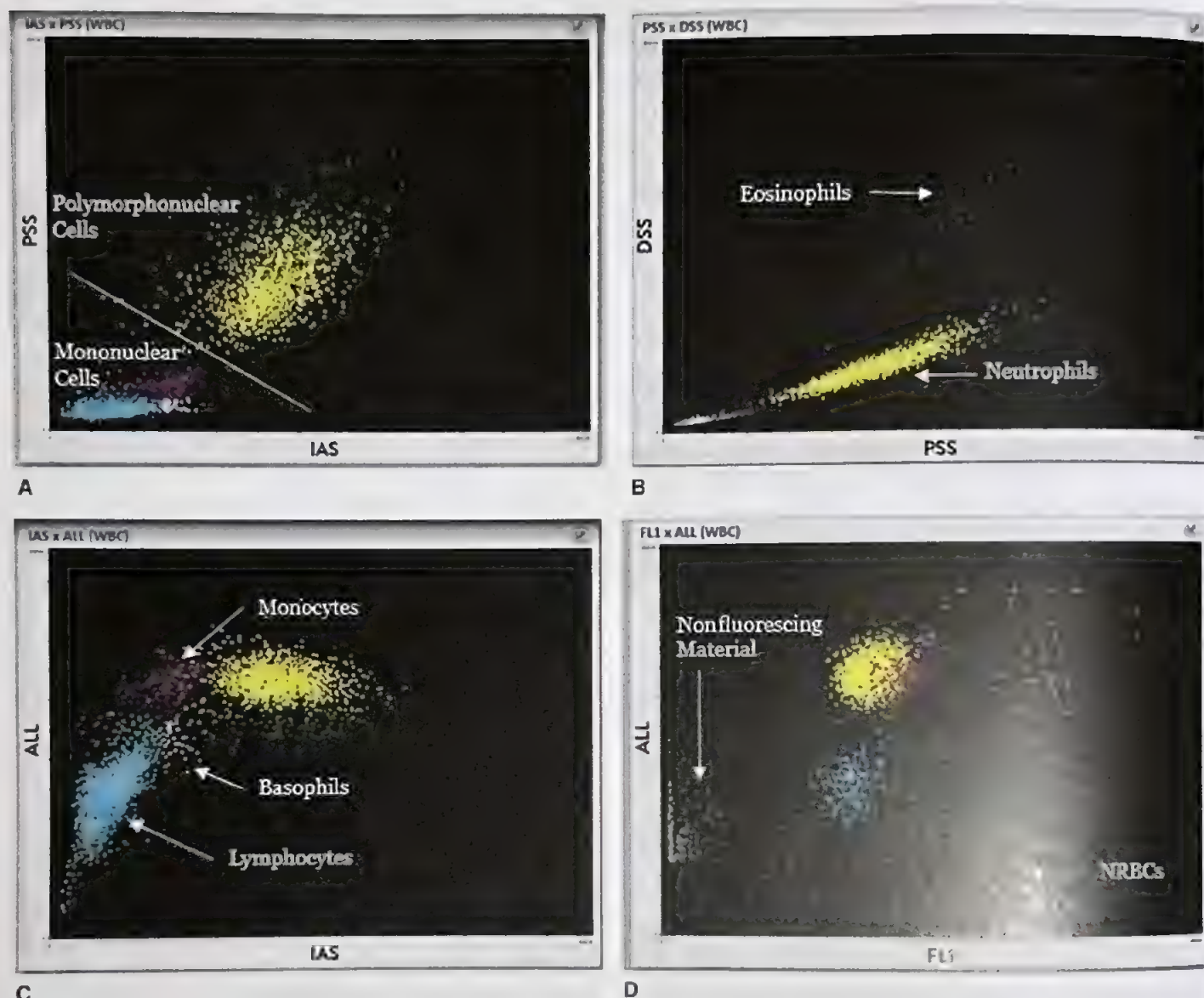


FIGURE 32-26 The separation of cells using scatterplots from the Alinity hq. **A.** Polymorphonuclear cells and mononuclear cells. **B.** Eosinophils and neutrophils. **C.** Monocytes, basophils, and lymphocytes. **D.** Nonfluorescing material and NRBCs (if present). (Courtesy of Abbott Laboratories, Abbott Park, IL.)



FIGURE 32-27 NRBC and NR/W parameters are included as part of every CBC. The WBC reagent contains a lyse and a nucleic acid-selective fluorescent dye. The lyse removes the membrane of the NRBC while leaving the WBCs intact and the dye fluoresces at 525 nm allowing for the differentiation of nucleated (WBC and NRBC) from nonnucleated cells. (Courtesy of Abbott Laboratories, Abbott Park, IL.)

Body Fluid Analysis

Despite the capability of Abbott's legacy Cell-Dyn series to provide accurate automated body fluid cell counts,³⁴ at the time of this publication, the Alinity h series analyzers are not currently approved to provide body fluid cell counts.⁹

However, preliminary internal evaluation studies have documented improved performance for body fluid cell counts using the dedicated body fluid application using less sample volume than previous models.³⁵

Additional Parameters

The cell-by-cell measurement of HGB concentration allows the creation of a CHC distribution curve (histogram), and from this, several Research Use Only parameters, including the CHC mean value (CHCM) and the Hemoglobin Distribution Width (HDW) can be derived. The CHCM is used to create a "calculated HGB" concentration using the MCV value and the RBC concentration. Although this is not a reportable parameter, it can be a helpful indicator of sample quality compared with measured HGB. If the measured and calculated HGB values differ by more than a defined threshold, the Alinity hq displays a "HGB Interf" flag alerting the user of the potential presence of sample interferences.

The HGB distribution curve is used to generate the % hypochromic (%HPO) and % spherocytic RBCs (%HPR) Research

TABLE 32-4 Morphological Flags on the Alinity hq

Morphological Flag	Display Name	Description
Platelet clump	PLT Clump	Indicates the presence of platelet clumps (aggregates).
Left shift	Left Shift	Indicates the presence of increased numbers of band neutrophils.
Blast	BLAST	Indicates the presence of blast cells.
Variant lymphocytes	VAR LYM	Indicates the presence of increased numbers of reactive, activated, or abnormal lymphocytes. It may also suggest the presence of blast cells.
RBC fragments	RBC Frag	Indicates the presence of RBC fragments or schistocytes.
Unlysed nonfluorescent RBCs	rstRBC	Indicates the presence of unlysed RBC (for example, target cells or sickle cells).
HGB interference	HGB Interf	Indicates a significant difference between photometric and cellular HGB results that is caused by the presence of interfering substances in the sample or by sample dispensing inaccuracy.

Source: Contributed by Abbott Hematology, used with permission.

Use Only parameters, based on preset thresholds. Studies have demonstrated that % hypochromic RBC is a sensitive parameter for detecting iron deficiency and functional iron deficiency^{37,38} and % spherocytic RBC can assist in screening for hereditary spherocytosis and its differentiation from autoimmune hemolytic anemias.³⁹ Other Research Use Only parameters available as part of RBC and PLT analysis include % microcytic RBCs (%MIC), % macrocytic RBCs (%MAC), and platelet (volume) distribution width (PDW).

Digital Morphology Analyzers: CellaVision System

Many laboratory automation platforms integrate automated hematology analyzers with automated slide makers and digital morphology analyzers to support the performance of WBC differentials in blood and body fluid samples. Development of early digital morphology analyzers began decades ago, but it did not become part of the regular laboratory workflow until the 2000s with the release of the first CellaVision digital morphology analyzer in 2001. While other analyzers do share a portion of the market (Sysmex DI-60, Vision Hema, Easy Cell, Next Slide, Cobas m511, HemaCam, and others), CellaVision analyzers (CellaVision® DM96, CellaVision® DM1200, CellaVision® DM9600) are the most popular and therefore, will be the focus of the literature discussed in this text.^{40,41} In the design of these systems, digital microscopes are coupled with cameras. The digital microscope can scan prepared and stained blood films, identify an appropriate area of the smear to evaluate, and the camera will capture images of red blood cells (50× oil immersion) and white blood cells (100× oil immersion) for analysis. Images are preclassified by the system using an artificial network that compares the captured images with a database of reference cells. The presorted cell populations can be reviewed on a computer screen by a qualified technologist for confirmation or reclassification if necessary.

Digitizing images for review offers several advantages, including the ability to conduct slide reviews or provide consultations from off-site or remote locations. Electronic archival of digital libraries can also facilitate technologist training

and competency evaluation.⁴² Research studies comparing the accuracy of morphology classification using the CellaVision system to results obtained from hematology analyzers and manual technologist review have demonstrated accurate classification of mature and precursor leukocytes.^{40,43} The system has also proven effective in the recognition of red blood cell morphology,⁴⁴ as a tool for platelet estimation,⁴⁵ and classification of cells in body fluids.⁴⁶

Despite automating a portion of the manual labor involved with differential reviews, the wide adoption and validation of digital morphology analyzers does not remove the necessity for review by a competent technologist. Recognition and classification of cells are limited to the comparative reference library within the analyzer's network and thus is subject to cases of classification error.⁴⁷ Comparative studies that have evaluated the performance of digital morphology analyzers integrated across different hematology analyzer manufacturer platforms also recognize the inability to standardize preanalytical, analytical, and postanalytical factors.^{40,41,48} These factors include natural variances between patient populations, concerns with sample integrity, differences between automated and manual blood smear preparation and staining, and application of laboratory decision rules for manual slide review. For these reasons, digital morphology analyzers will remain a powerful laboratory tool but cannot replace the manual intervention required of competent hematology laboratory technologists.

Quality Control and Quality Assurance Measures for Automated Complete Blood Count Instruments

The Clinical Laboratory Improvement Amendments of 1988 and accrediting agencies such as the College of American Pathologists (CAP) and the Joint Commission on Accreditation of Healthcare Organizations (JCAHO) require laboratories to establish a system for quality control and quality assurance.⁴⁹ This mechanism safeguards the quality of the results and services a laboratory can provide. The system is composed of several elements that ensure quality through the preanalytic, analytic, and postanalytic phase of

testing. For automated hematology analyzers, quality assurance includes validation procedures, calibration and calibration verification, quality control, instrument maintenance, proficiency testing, and result reporting procedures.

Quality Control Procedures

QC measures for CBC instruments involve the analysis of stabilized control material to evaluate the accuracy and precision of the instrument. Routinely, control materials are assayed by the manufacturer and have target means and ranges established for each lot number. Despite these established means and ranges, laboratories must verify the manufacturer's ranges or establish their own ranges before using a new lot of control material to monitor and check instrument performance. Once means and ranges are established, at least two levels of control material should be analyzed every 8 hours during each day of patient testing. Analyzers with reticulocyte and body fluid counting capabilities will require additional QC measures. QC results should be evaluated daily for acceptability and periodically thereafter (i.e., weekly or monthly) utilizing statistics such as coefficient of variation (CV) and standard deviation (SD) and other tools such as Westgard rules or Levy-Jennings charts to detect imprecision. System performance can also be monitored during routine analysis of patient specimens using moving average programs (including X-B). In addition, laboratories must also have procedures in place that outline what actions personnel should take when daily QC is unacceptable or when QC statistics have changed significantly. Quite often, manufacturers of automated hematology analyzers offer interlaboratory peer QC programs to provide added quality assurance. Subscribers of these programs submit QC results on a monthly basis and in return they receive a comprehensive report that compares their results against those of peer laboratories using the same lot of control material. Real-time monitoring of results off-site can also permit manufacturers to suggest preventative maintenance.

Quality Assurance Measures

Successful participation in an external proficiency testing program is an essential quality assurance action required to meet accreditation requirements for CLIA and other agencies. Proficiency testing programs provide blind samples to laboratories for scheduled testing two or three times per year. Laboratories must handle and test samples in the same manner as patient testing. Proficiency testing results are submitted to the testing provider for analysis. After the testing window closes, each participating laboratory receives a comparison report that evaluates their performance against other participants utilizing the same technology. Evaluation summaries provide independent and objective assessment of performance and can be invaluable tools for detecting problems in instrumentation and identifying the need for additional employee training.

Also important in ensuring quality results is instrument maintenance. Maintenance procedures and required frequency are analyzer and manufacturer dependent. Preventative maintenance procedures are developed by the manufacturer and are designed to keep the instrument functioning properly. Maintenance can range from simple actions such as flushing the tubing on a daily basis to more in-depth procedures that are performed less

frequently by manufacturer technical support personnel. Unscheduled maintenance may also be required to troubleshoot spurious patient results or resolve automated operational error codes.

Before the implementation of an automated hematology system, laboratories must validate the instrument's performance. This process involves verifying the accuracy and precision of the instrument, performing linearity studies to verify or establish the instrument's analytic measurement range, and correlation studies that compare the new method to the current method or other reference standard. Calibration procedures are a set of operations established by the manufacturer to ensure an instrument consistently yields accurate results. For CBC instruments, manufactured stabilized whole blood calibrators are used to adjust and verify the accuracy of the measuring system. In addition, multiple analyses of fresh whole blood specimens are tested to ensure reproducibility. Calibration verification is performed immediately after calibration and periodically thereafter to check the accuracy of the calibration. Frequency of calibration and calibration verification depends on the instrument manufacturer's recommendations and accreditation agency requirements. In addition, laboratories must also establish their own criteria for calibration and calibration verification. Examples where calibration might be required include unusual trends in quality control results or after major system maintenance.

Result Verification and Decision Rules

Establishing procedures for evaluating and reporting automated CBC results helps maximize efficiency and prevents the reporting of erroneous results. New advances such as user-friendly computer software and enhanced software capabilities that improve cell population separation, automated reticulocyte counts, bar code sample identification, closed tube sampling, on board sample mixing and slide making, and greater sample throughput have improved laboratory safety, productivity, efficiency, and workflow. Moreover, these advanced features contribute to cost-effective laboratory operations and efforts to preserve quality patient care in the managed care environment.

Because current automated analyzers with histogram differential capabilities generate comprehensive interpretive reports containing detailed information about abnormalities, careful evaluation of the data can provide an efficient screening tool. Automated results can direct the technologist to focus on specific problems and troubleshoot for spurious results that may arise in either quantitative or qualitative parameters. Artifactual hematologic abnormalities in laboratory test results can lead to serious complications as a result of an incorrect diagnosis and inappropriate treatment based on undetected, technically induced errors.⁵⁰ A review of the literature suggests that the newer hematology analyzers have become increasingly sophisticated and the chances of advanced automated blood cell analyzers missing a significant abnormality are small. Nevertheless, despite advanced technologies currently in use, artifactual and spurious results still occur⁵⁰⁻⁵⁴ (Table 32-9). The awareness of instrument limitations, appropriate troubleshooting, and judicious use of reflex testing protocols by laboratory professionals can prevent the reporting of erroneous results and incorrect treatment of patients with suspected hematologic disorders.

TABLE 32-9 Spurious Hematology Results: Errors in Measurement and Sample Integrity

Analyzer Value	False Decrease	False Increase
WBC	Cold agglutinins Leukoagglutination EDTA induced aggregation (glycoprotein IIb/IIIa auto-Ab mediated) Dilution	Cryoglobulins microorganisms present NRBCs Lyse resistant RBCs (ex. sickle cells and target cells) PLT aggregation, clumping
Neutrophils	Dysplastic granulocytes (hypolobular/hypogranular) Degenerated cells are not counted	Rare
Lymphocytes	Rare	NRBCs counted as lymphocytes Dysplastic granulocytes misclassified Hypergranulated basophils
Monocytes	Rare	Atypical lymphocytes, blasts, or dysplastic granulocytes misclassified
Eosinophils	Degranulation Dysplasia (hypogranulation)	Cell inclusions cause misclassification (ex. hemosiderin, malarial parasites)
Basophils	Dysplasia (hypogranulation)	Poor mixing Abnormal granulation
RBC	Cold agglutinins Dilution	Giant PLTs Large RBC fragments
HGB	Dilution Elevated sulphemoglobin	Turbidity (ex. hyperlipidemia, contamination, hypergamma globulinemia, cryoglobulinemia) Extreme leukocytosis Extreme hemolysis (hemoglobinemia) Elevated carboxyhemoglobin
HCT	Errors in RBC or MCV measurement	Errors in RBC or MCV measurement
MCV	Hypo-osmolar cells dehydrate in diluent Giant PLTs	Cold agglutinins Hyper-osmolar cells overhydrate and swell in diluent (ex. hyperglycemia, hyponatremia, dehydration)
MCH	Errors in HGB or RBC measurement	Cold agglutinins
MCHC	Errors in HGB measurement or HCT calculation (RBC or MCV measurement errors)	Errors in HGB measurement or HCT calculation (RBC or MCV measurement errors)
PLT	Cold agglutinins Giant PLTs PLT-WBC rosettes (Satellitism) PLT-WBC aggregation EDTA induced aggregation (glycoprotein IIb/IIIa auto-Ab mediated) Clotted sample Dilution	Cryoglobulins Fragmented RBCs Cytoplasmic fragments Extreme microcytosis Microorganisms present

Most laboratories use a standardized set of decision rules to decide if analyzer results should be automatically verified or if the results require further evaluation. These rules are designed to identify abnormal or unexpected patient results and improve the slide review rate by eliminating unnecessary slide reviews. Decision rules can be applied as patient results are transferred to the Laboratory Information System (LIS) or middleware. Additional on-board decision rules embedded into the analyzer software can automatically reflex or repeat sample testing. In fully integrated analyzers, this can include the automatic preparation of stained smears for review.

Consensus rules for slide review were originally published by the International Society of Laboratory Hematology (ISLH) in 2005 to assist laboratories in refining rules for slide review.⁵⁵ Examples include action limits on RDW, HGB, and RBC parameters to trigger a morphological evaluation of blood films, limits on RBC indices to identify causes for interferences such as lipemia or hemolysis, and limits on platelet counts to recognize platelet clumping or sample clotting. Included within the criteria for each parameter are suggestions that outline necessary corrective actions to take when results exceed prescribed limits (Table 32-10). Although the ISLH review criteria have been widely validated, variations

TABLE 32-16 International Consensus Group for Hematology Review Consensus Rules

Rule	Parameter	Primary	and/or	Secondary	and/or	Tertiary	and/or	Fourth	Action 1	Action 2	Action 3
1	Neonate	First sample							Slide review		
2	WBC, RBC, HGB, PLT, Retics	Exceeds linearity							Dilute sample and rerun		
3	WBC, PLT	Lower than lab-verified instrument linearity							Follow lab SOP		
4	WBC, RBC, HGB, PLT	Vote Out							Check sample for clot	Rerun sample	If persists, perform alternate counting method
5	WBC	<4.0 OR >30.0	and	first time					Slide review		
6	WBC	<4.0 OR >30.0	and	delta failed	and	within 3 days			Slide review		
7	PLT	<100 OR >1000	and	first time					Slide review		
8	PLT	Any value	and	delta check fail					Slide review		
9	HGB	<7g/dL or >2 g/dL above upper reference range for age and sex	and	first time					Slide review	Verify sample integrity if indicated	
10	MCV	<75 fL or >105 fL (Adult)	and	first time	and	specimen is < 24 hours old			Slide review		
11	MCV	>105 fL	and	Adult	and	specimen is >24 hrs old			Slide review for macrocytic associated changes	Request fresh sample if NO macrocytic associated changes seen	Report with comment if fresh sample is not available
12	MCV	Any value	and	delta fails	and	specimen is <24 hours old			Verify sample integrity/identity		
13	MCHC	≥2 units above upper limit of reference range							Check for lipemia, hemolysis, RBC agglutination, spherocytes		
14	MCHC	<30	and	normal/high MCV					Investigate possible IV contamination or other sample specific cause		
15	RDW	>22	and	first time					Slide review		
Differential											
16	No diff or Incomplete diff								Manual diff and slide review		
17	Neut #	<1.0 or >20.0	and	first time					Slide review		
18	Lymph #	>5.0 (adult) or >7.0 (<12 years old)	and	first time					Slide review		
19	Mono #	>1.5 (adult) or >3.0 (<12 years old)	and	first time					Slide review		
20	Eos #	>2.0	and	first time					Slide review		
21	Baso #	>0.5	and	first time					Slide review		
22	NRBC #/RETICS	Any value	and	first time					Slide review		
23	Retic Absolute #	>0.100	and	first time					Slide review		
Suspect Flags											
24	Suspect flag (except ImmG/Band)	Flag +	and	first time	and	Adult			Slide review		
25	Suspect flag	Flag +	and	first time	and	Child			Slide review		
26	WBC unreliability Flag	Flag +	any						Check sample integrity and rerun sample	If persists, review instrument output	Slide review with manual diff if indicated
27	RBC fragment	Flag +	any						Slide review		
28	Dimorphic RBC	Flag +	and	first time					Slide review		
29	Lyse resistant RBC	Flag +	any						Review WBC histogram/cytogram	Validate by lab SOP (consider incorrect retic count)	E.g., review smear for abnormal RBC morphology
30	PLT clump flag	Any count							Check sample for clots	Slide review (PLT estimate)	If clumps persist, follow lab SOP

Continued

TABLE 32-10 International Consensus Group for Hematology Review Consensus Rules—conr'd

Rule	Parameter	Primary	and/or	Secondary	and/or	Tertiary	and/or	Fourth	Action 1	Action 2	Action 3
31	Platelet flags	PLT & MPV flags except plt clumps							Slide review		
32	Immature granulocyte flag	Flag +	and	first time					Slide review		
33	Immature granulocyte flag	Flag +	and	Previous confirmed result	and	positive delta fail for WBC			Slide review		
34	Left shift flag	Flag +							Follow lab SOP		
35	Atypical/variant lymphs	Flag +	and	first time					Slide review		
36	Atypical/variant lymphs	Flag +	and	Previous confirmed result	and	positive delta fail for WBC			Slide review		
37	Blast flag	Flag +	and	first time					Slide review		
38	Blast flag	Flag +	and	Previous confirmed result	and	delta pass or negative delta for WBC	and	within 3-7 days	Follow lab SOP		
39	Blast flag	Flag +	and	Previous confirmed result	and	positive delta fail for WBC			Slide review		
40	NRBC flag	Flag +							Slide review	If positive, enumerate NRBC count, correct WBC if appropriate	
41	Retics	Abnormal pattern							Look at instrument output	Repeat if aspiration problem	If persists, review slide

Source: Consensus Guidelines available on the International Society for Laboratory Hematology Website (www.islh.org). Accessed on November 27, 2006.

in instrument technology and patient populations have led regulatory agencies and others to suggest the need for local verification of these standards in a laboratory setting before implementation.^{56,57}

CRITICAL THINKING QUESTION

32-3 How often should quality control samples be tested on hematology instruments?

SUMMARY CHART

- Automated cell counts and differential analysis is an important screening tool in the laboratory that can require confirmation using manual procedures.
- Automated differential analysis is currently based on quantitation, evaluation of cell volume, cell light scattering, cytochemistry, and fluorescence.
- The Coulter Principle of electronic impedance is the gold standard technology used to count and size cells.
- Red cell histograms quantify red blood cells and calculate cell size (MCV) and red cell distribution width (RDW) as well as measure hemoglobin, including mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC).
- Advanced Red Cell parameters such as Ret-He, IRF, and CHr provide additional information regarding reticulocyte quality and maturity and are more sensitive than the reticulocyte count in the assessment of bone marrow activity.
- Platelet histograms quantify and display characteristics of size and uniformity platelets.
- Leukocyte histograms quantify and separate white blood cell subpopulations.
- Coulter VCS technology uses three independent energy sources, including electrical impedance to measure volume, light-scattering characteristics for determination of cell structure and shape, and conductivity (RF) for differentiation of white blood cell subpopulations.
- Siemens automated blood analyzers are based on principles of optical flow cytometry, cytochemistry, and light scattering.
- Sysmex automated blood analyzers use direct current (DC), hydrodynamic focused flow impedance, and fluorescent flow cytometry to size and count blood cells. Flow cytometry is used to differentiate WBC subpopulations.
- Abbott analyzers use MAPSS (Multi-Angle Polarized Scatter Separation) technology to count and differentiate WBCs.
- Analyzers will flag sample abnormalities to alert the technologist of results in need of further investigation or confirmation by reviewing the peripheral blood smear.
- Digital morphology analyzers such as the CellaVision can scan peripheral blood smears, capture digital images, and preclassify cells for review by a technologist.
- Federal regulations require laboratories to develop a quality assurance system.
- Quality control and analyzer maintenance procedures are analyzer dependent.
- Each institution should establish criteria for the review of automated results.

CASE STUDIES

Please see the Case Studies with accompanying illustrations at www.fadavis.com.

Acknowledgments

The authors acknowledge and thank Beckman Coulter Inc., Siemens Healthcare Diagnostics, Sysmex Corporation, and Abbott Hematology for their contributions to this chapter.

REVIEW QUESTIONS

1. What is the main use of leukocyte histograms and scatterplots?
 - a. Screening for hematology parameters
 - b. Primarily a research tool
 - c. Replace the manual differential
 - d. Measuring WBC function
2. What information from the CBC is illustrated by a histogram?
 - a. Specific cell morphology
 - b. Exact number of cells
 - c. Average cell size
 - d. Cell differential
3. Which of the circumstances below can lead to inaccurate analyzer results?
 - a. High WBC count
 - b. NRBCs
 - c. Low hematocrit
 - d. High neutrophils
4. What does an RBC histogram distribution curve that is shifted to the right indicate?
 - a. Macrocytosis
 - b. Anisocytosis
 - c. Microcytosis
 - d. Spherocytosis
5. What does an RBC histogram distribution curve that is shifted to the left indicate?
 - a. Macrocytosis
 - b. Anisocytosis
 - c. Microcytosis
 - d. Spherocytosis
6. The RDW represents a parameter that quantifies relative:
 - a. Macrocytosis
 - b. Anisocytosis
 - c. Platelet size
 - d. Microcytosis
7. What do the MPV and PDW parameters describe?
 - a. White blood cell size
 - b. Red blood cell size
 - c. Platelet size
 - d. Multiple cell size
8. What does an increased IPF or reticulated platelet value best indicate?
 - a. Bone marrow failure
 - b. Increased platelet production
 - c. Increased platelet destruction
 - d. Immature granulocytes
9. Which of the following best describes the principle of the white cell differential of Coulter DxH Series instruments?
 - a. Laser and light scatter
 - b. Volume, cell size, and cytochemistry
 - c. Volume, conductivity, and light scatter
 - d. Laser and immunofluorescence
10. Which of the following best describes the principle of the Siemens ADVIA 120/2120 technology?
 - a. Electrical impedance, volume, and conductivity
 - b. Flow cytometry, cytochemistry, and light scatter
 - c. Chemiluminescence and laser cell counting
 - d. Cell volume and light scatter
11. What best characterizes the principle of Sysmex XN-Series analyzers?
 - a. Impedance and light scatter
 - b. Cytochemistry and flow cytometry
 - c. Impedance and fluorescent flow cytometry
 - d. Conductivity and volume
12. Which best describes the differential principle of the Abbott Alinity iq?
 - a. VCS
 - b. MAPSS
 - c. Impedance
 - d. Cytochemistry
13. How often is quality control recommended for hematology analyzers?
 - a. One level each day of testing
 - b. Two levels each day of testing
 - c. One level every 8 hours, each day of testing
 - d. Two levels every 8 hours, each day of testing
14. What should be the basis for the decision to accept an automated leukocyte histogram differential or to perform a conventional manual differential?
 - a. Each laboratory's established criteria
 - b. Availability of qualified technologists
 - c. Laboratory workload
 - d. Manufacturer's recommendation

See answers at the back of this book.

CHAPTER 33

Coagulation Methods

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CHAPTER OUTLINE

Platelet Function Instrumentation and Tests

- Method 33-1. Bleeding Time
- Method 33-2. Closure Time—PFA-100® (Siemens)
- Method 33-3. Platelet Aggregation

Coagulation Instrumentation

- General Types of Coagulation Instrumentation
- Methods of Endpoint Detection
- Complete Hemastasis Evaluation

Coagulation Screening Tests

- Method 33-4. Activated Partial Thromboplastin Time
- Method 33-5. One-Stage Prothrombin Time (Quick)
- Method 33-6. Thrombin Time
- Method 33-7. Mixing Studies—aPTT or PT 1:1 Mix

Coagulation Factor Assays

- Method 33-8. One-Stage Quantitative Assay Method for Factors II, V, VII, and X
- Method 33-9. One-Stage Quantitative Assay Method for Factors VIII, IX, XI, and XII
- Method 33-10: Factor XII. Chromogenic Assay (Activity)

Coagulation Inhibitors

- Tests to Monitor Anticoagulant Therapy
- Monitoring Anticoagulant Therapy With Coagulation Screening Assays
- Method 33-11. Anti-FXa Assay (Heparin Activity)

Monitoring Direct Thrombin Inhibitors

Tests to Measure Fibrin Formation

- Method 33-12. Reptilase Time
- Method 33-13. Fibrinogen Activity

Tests for von Willebrand's Disease

- Method 33-14. von Willebrand Factor Antigen
- Method 33-15. von Willebrand Factor Activity (vWF:RCO, Ristocetin Cofactor)
- von Willebrand Collagen Binding Activity
- von Willebrand Factor Multimer Analysis
- Molecular Analysis in vWD

Tests to Assess Hereditary Thrombotic Risk

- Method 33-16. Activated Protein C Resistance/Factor V Leiden

Antithrombin Assays

- Method 33-17. Antithrombin Functional Assay (Activity)—Chromogenic Substrate Assay
- Method 33-18. Antithrombin Immunological Assay (Antigen)—Microlatex Particle Immunological Assay

Protein C Assays

- Method 33-19. Protein C Immunological Assay (Antigen)
- Method 33-20. Protein C Functional Assays (Activity)—Chromogenic Substrate Assay

Method 33-21: Protein C Clot-Based Assay

Protein S Assays

- Method 33-22. Protein S Functional Assay (Activity)—Clotting Assay
- Protein S Immunological Assay (Antigen)

Prothrombin G20210A (Factor II) Mutation

Tests for the Evaluation of Lupus Anticoagulants

Confirmatory Tests for Lupus Anticoagulants

- Method 33-23. Platelet Neutralization Procedure
- Hexagonal Phospholipid Neutralization Assay
- Anti-Phospholipid Assays

Tests for Fibrinolysis

- D-Dimer Quantitative Test
- Method 33-24. Euglobulin Lysis Time
- Method 33-25. Fibrin Degradation Products: Latex Agglutination Method

Markers of Coagulation Activation and Thrombin Generation

Summary Chart

Case Study 33-1

Case Study 33-2

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 33-1 Correlate platelet function tests with the specific hemostatic mechanism being assessed.
- 33-2 Describe the challenges of utilizing the bleeding time test in primary hemostasis screening.
- 33-3 Explain why the activated partial thromboplastin time and the prothrombin time are considered screening tests and not diagnostic tests.

- 33-4 Determine the need for a mixing study based on patient history and activated partial thromboplastin time and prothrombin time test results.
- 33-5 Assess activated partial thromboplastin time mixing study results consistent with factor deficiencies versus inhibitors.
- 33-6 Categorize factor assays into those utilizing the prothrombin time and those utilizing the activated partial thromboplastin time as the base testing method.

LEARNING OBJECTIVES—cont'd

- 33-7** Link inhibitor assays to the types of patients that may develop associated inhibitors.
- 33-8** Assess the use of the thrombin time and reptilase time.
- 33-9** Contrast tests utilized to monitor heparin versus those to monitor warfarin.
- 33-10** Explain the importance of the von Willebrand factor molecule.
- 33-11** Differentiate tests for von Willebrand's disease as either quantitative or qualitative.
- 33-12** Evaluate hereditary and acquired forms of thrombosis and the tests used to assess them.
- 33-13** List criteria for the laboratory diagnosis of lupus anticoagulants.
- 33-14** Interpret D-dimer assay results and correlate results to clinical diagnosis.
- 33-15** Describe the three diagnostic applications of coagulation activation.
- 33-16** Compare fully automated, semiautomated, and manual methods of coagulation testing.
- 33-17** Describe the different endpoint detection methodologies employed by coagulation instrumentation.

Platelet function tests and coagulation instrumentation are coagulation methods necessary for assessment of primary and secondary hemostasis mechanisms, as well as adequate fibrinolysis and the evaluation of potential factor, protein, and inhibitor deficiencies. These methods contribute to the capability of understanding a patient's hemostatic condition. Coagulation methods include a collection of fully automated, semiautomated, and manual analysis methods. The following are general considerations regarding coagulation procedures:

- Sodium citrate is the anticoagulant used for routine coagulation procedures (3.2%, 0.109 M). Other anticoagulants such as ethylene diaminetetraacetic acid (EDTA), heparin, or oxalate are unacceptable.
- The ratio of blood to anticoagulant should be 9:1. A disproportion of blood to anticoagulant is seen in patients with polycythemia.¹ When the hematocrit is higher than 55%, the amount of sodium citrate used should be adjusted (decreased) according to the following calculation:

$$C = 1.85 \times 10^{-3} \times (100 - H) \times V$$

where

C = volume of sodium citrate in milliliters,

H = hematocrit in percent, and

V = volume of whole blood in milliliters.

- Each laboratory should develop its own normal values reflecting the methodology, reagents, instrumentation, and patient population.
- The technique used in obtaining and processing the patient's blood sample and the conditions under which samples are stored or transported determine the integrity of the final test result. Traumatic venipuncture may result in activation of coagulation factors, and improper storage conditions may result in the deterioration or (in the case of factor VII) activation of coagulation factors.
- Most clot-based coagulation activity procedures are performed on plasma, thereby requiring the addition of calcium to perform the tests because the anticoagulant used, sodium citrate, binds free calcium.
- Clot-based methodologies are usually performed at $37^{\circ}\text{C} \pm 1^{\circ}$.

- Immunological assays are currently available for a number of coagulation factors, inhibitors, and proteins involved in fibrinolysis. Because these assays determine the presence or absence of proteins and not their biological activity, functional testing should be performed in addition, when possible.

ADVANCED CONTENT

In the past, evaluation of hemostasis relied on traditional procedures based on the detection of clot formation. The innovation of enzyme-specific synthetic substrates has had a great effect on the field of hemostasis. Knowledge of molecular structures for different enzymes and the cleavage points of their corresponding substrate has led to the development of synthetic substrates that are cleaved by a single factor enzyme. Cleavage of synthetic substrates occurs when the amino acids of the substrates on the molecule fit into the active sites and the binding sites of the enzyme. All synthetic substrates rely on cleavage of the peptide by their specific enzymes, releasing a chromogenic complex such as *para*-nitroaniline or a fluorogenic complex such as aminoisophthalic acid dimethyl ester (AIE), which may be detected and measured by means of a spectrophotometer or fluorimeter. Various synthetic chromogenic and fluorogenic assays exist for the evaluation of plasmin, plasminogen activator, alpha-2 (α_2 -antiplasmin, kallikrein, antithrombin [AT-III]), factor Xa, thrombin, and several other serine proteases, making these assays applicable for routine laboratory testing. Procedures using synthetic substrates have certain advantages over the traditional clot formation techniques. They can be performed rapidly, are sensitive, allow a greater degree of standardization, require smaller sample volumes, and are well suited for automation. Synthetic substrates facilitate measuring the activity of clotting factors and their inhibitors. Often, individual stages of a reaction can be assayed without having to observe the entire cascade of the clotting process.

Depending on testing methodology and/or instrumentation requirements, sodium citrate samples can be used as whole blood, platelet-poor plasma, or platelet-rich plasma. In general, platelet testing involves either whole blood or platelet-rich plasma, while coagulation testing requires either whole blood or platelet-poor plasma. If the type of plasma is not specified, platelet-poor plasma is expected to be used. Differences between the two types of plasma depend on the centrifugation time and speed. Platelet-poor plasma is centrifuged for 10 minutes at $2,500 \times g$, while platelet-rich plasma is centrifuged for 30 minutes at $50 \times g$.

Platelet Function Instrumentation and Tests

The contribution of platelets to the hemostatic mechanism is through adhesion and aggregation, which is known as *primary hemostasis*. Adhesion is defined as the platelet's ability to stick to a surface, such as subendothelial collagen. Aggregation assesses the ability of the platelets to stick to each other. For this to occur, platelets must be able to secrete the contents of their organelles (α granules, dense bodies), which contribute to additional platelet activation and aggregation. Platelet function testing measures or monitors the platelet's ability to adhere and aggregate and has historically presented a challenge for the clinical laboratory because of the lack of reliable, accurate, and easy-to-perform testing methodologies.

In the laboratory, the **Bleeding Time Test** has historically been used to measure platelet adhesion, whereas aggregation studies have been used to observe platelet secretion and the ability to stick to each other. The bleeding time is a screening test performed by phlebotomists or nurses at the patient's bedside. A wide range of testing variability is based on skin thickness, puncture depth, temperature, and procedural variances, which can provide unreliable results. Consequently, the PFA-100[®] offers a standardized option as a screening test for congenital and acquired primary hemostatic disorders in laboratories.²

The PFA-100[®] closure time utilizes optical aggregometry as a screening test for platelet function and is performed on citrated whole blood. It evaluates the ability of platelets to interact with collagen and ADP or epinephrine in a capillary setting to close the lumen while under flow conditions similar to that of the capillary vasculature.³ Though more standardized than the bleeding time, it must still be considered a screening test that is not 100% sensitive to platelet defects; thus light transmission platelet aggregometry (LTA) is regarded as the gold standard in platelet function testing.²

Platelet aggregation assessment provides an opportunity to reduce variability and provide a more sensitive assessment of platelet function. Optical and impedance aggregometry allow the use of a small volume of whole blood or platelet-rich plasma to provide an assessment of platelet aggregation in a more physiological environment. Impedance aggregometry exploits the ability of platelets to adhere to artificial surfaces. As the platelets aggregate on two agonist-coated electrode surfaces, the current intensity

diminishes reflecting increasing electrical impedance. The rate of electrical impedance reflects platelet function. In **optical aggregometry** the sample is dispersed through an agonist-coated membrane at a high shear rate. As platelets aggregate on the membrane, the membrane flow rate diminishes. The cessation of flow is defined as the "closure time."

LTA requires platelet-rich plasma to evaluate platelet to platelet clump formation *in vitro* in a glycoprotein IIb/IIIa-dependent manner. Various agonists are added to initiate aggregation. The platelet aggregation rate and maximum percentage of aggregation are measured via the increasing light transmission through the optically dense samples as face platelets are employed in the forming aggregate employing a photometer. Software embedded in analyzers determines the slope of the curve, the maximum state of aggregation and the lag phase of the curve. Because evaluation of platelet function can be of critical importance in the hemostatic management of a patient, especially for those undergoing surgical procedures with inherited platelet disorders and inherited platelet function disorders, improved ability to assess platelet function in a timely and efficient manner is essential.⁴

Method 33-1: Bleeding Time

The bleeding time test assesses platelet function *in vivo* and is used as a screening procedure to detect both congenital and acquired disorders of platelet function and von Willebrand's disease. The bleeding time may be measured as part of a diagnostic work-up of a quantitative or qualitative platelet disorder, or von Willebrand's disease, but it is not able to distinguish overall bleeding risk or a coagulation factor deficiency.

The bleeding time response is inversely related to platelet count. The use of aspirin, aspirin-containing drugs, nonsteroidal anti-inflammatory drugs, and antihistamines causes a prolonged bleeding time. The patient should be instructed not to take any aspirin or drugs containing aspirin for 1 week before the test is performed. Several devices are available for making standardized incisions when performing a bleeding time.

Today the bleeding time test is rarely used due to variability in incisions depth, blood vessel involvement, and in interpretation. Advances in technology provide alternate testing platforms with proven higher sensitivity and specificity.

Method 33-2: Closure Time—PFA-100[®] (Siemens)

The closure time test screens platelets for their ability to adhere and aggregate under capillary flow conditions, similar to those found *in vivo*. When circulating *in vivo*, platelet motion and position in the vessel are affected by the flow created by the concave red blood cells, a high shear condition, and platelets are directed toward the side of the vessel. In this assay, platelet activation is affected by the red blood cell and platelet counts and may be prolonged with a hematocrit less than 35% or a platelet count of less than $150,000/\mu\text{L}$.⁵

Method 33-3: Platelet Aggregation

Platelets function in primary hemostasis by forming an initial platelet plug at the site of vascular injury. The phenomenon occurs partly through the ability of platelets to adhere

METHOD 33-1 Bleeding Time (Obsolete and Rarely Performed)

Principle	Bleeding time is defined as the time taken for a standardized skin incision to stop bleeding. Upon vessel injury, platelets adhere to collagen exposed in the sub endothelium with assistance from von Willebrand factor and form a homeostatic platelet plug. The bleeding time test measures the ability of these platelets to arrest bleeding and, therefore, measures platelet number and function. Capillary contractility and both the intrinsic and extrinsic systems of coagulation function in a minor capacity in the bleeding time. The bleeding time primarily assesses in vivo platelet function.
Specimen	Capillary blood in vivo
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	Reference range is approximately 2.5 to 9.5 minutes and should be established by each laboratory. Prolonged bleeding times are found in the following situations: <ul style="list-style-type: none"> • Thrombocytopenia⁵ (platelet count less than 50,000/μL) • Inherited platelet dysfunction (Bernard Soulier syndrome or Glanzmann's thrombasthenia, storage pool disease) • After administration of aspirin or aspirin-containing drugs • After administration of other drugs that inhibit platelet function such as antihistamines • Von Willebrand disease
Limitations	If the incision fails to bleed or if a small vein is cut, disregard the bleeding time of the incision and repeat the test. In older patients, bleeding may only occur subcutaneously (under the skin). Obtaining an accurate bleeding time in these patients may not be possible.
Reporting Results	Results are reported in minutes
Reference Ranges	Bleeding time 2.5 to 9.5 minutes
Notes	<i>Caution:</i> Patients should be informed that, with any bleeding time procedure, the possibility of faint scarring exists. Keloid formation, although rare, can occur in certain patients.

METHOD 33-2 Closure Time—PFA-100® (Siemens)

Principle	Citrated whole blood is added to a cartridge fitted with capillary tubes coated with either collagen/ADP, or collagen/epinephrine. The cartridge is placed into the PFA-100 analyzer and suction is applied to slowly aspirate the whole blood sample through the capillary coated with the antagonists. As platelets are exposed to the collagen surface, they begin to adhere and then aggregate. The time it takes for the platelet to close the aperture of the capillary tube is detected and recorded as the closure time.
Specimen	Citrated whole blood
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	This assay is an effective method to screen for von Willebrand's disease, an important cause of menorrhagia, gingival bleeding, and epistaxis. The closure time can also distinguish between drug-related platelet inhibition and hereditary platelet defects (Table 33-1). ⁶
Limitations	Falsely decrease results can be acquired when platelet counts are <50 K/ μ L.
Reporting Results	Closure time is reported in seconds
Reference Ranges	COLL/EPI 80 to 190 seconds COLL/ADP 60 to 122 seconds

TABLE 33-1 Interpretation of PFA-100® Closure Time Results

Closure Time	ADP/Collagen	Epinephrine/Collagen
Normal	Normal	Normal
Platelet defect/von Willebrand's disease	Prolonged	Prolonged
Aspirin/drug-like effect	normal	Prolonged

to one another, a process known as *aggregation*. Substances that can induce platelet aggregation include collagen, ADP, epinephrine, thrombin, serotonin, arachidonic acid, the antibiotic ristocetin, snake venoms, antigen-antibody complexes, soluble fibrin monomer complexes, and **fibrin(ogen) degradation products (FDPs)**. These aggregating agents induce platelet aggregation, cause platelets to release endogenous ADP, or both. Platelet aggregation is an essential part of the investigation of any patient with a suspected platelet dysfunction.

METHOD 33-1 Platelet Aggregation

Principle

Platelet aggregation is studied by means of a platelet aggregometer, a photo-optical instrument connected to a chart recorder. Platelet-rich plasma, which is turbid in appearance, is placed in a cuvette, warmed to 37°C in the heating block of the instrument, and stirred via a small magnetic bar. Baseline light transmittance through the platelet-rich plasma is recorded. The addition of an agonist causes the formation of larger platelet aggregates with a corresponding increase in light transmittance, because of a clearing in the platelet-rich plasma. The change in light transmittance is converted to electronic signals and recorded. Other models of aggregometers use the principle of electrical impedance to determine formation of platelet aggregates. As platelets aggregate, they coat an electrode, impeding the electrical current through the analyzer. Certain models of aggregometers have the ability to measure the release of ATP from platelets using luminescence technology in either platelet-rich plasma or whole blood. A luminescent chemical is added to the reaction that releases a flash of light when stimulated by ATP energy release from the dense granules of the platelet. This modification of aggregation is particularly sensitive to ATP release and is a sensitive measure of platelet activation.

Specimen

Sodium citrate anticoagulated platelet-rich plasma

Procedure

See the required equipment and steps for this procedure at www.fadavis.com

Interpretation

Platelet aggregation occurs as a two-step process, known as *primary* and *secondary waves of aggregation*. The primary wave of aggregation is observed when platelets adhere to one another in the presence of an external agent (agonist) such as ADP, epinephrine, or ristocetin. Secondary aggregation is characterized as the aggregation that occurs after the platelets have been stimulated to secrete the substances contained in their organelles. It should be noted that some agonists will stimulate primary aggregation, and some will stimulate secondary aggregation. Others will stimulate both primary and secondary aggregation, yielding a "biphasic" aggregation curve (Fig. 33-1). In addition, different concentrations of the same agonist can produce varying patterns of primary and secondary aggregation. For example, low concentrations of ADP induce biphasic aggregation (i.e., both a primary and a secondary wave of aggregation); very low concentrations of ADP (1.5 mcg/mL final concentration) induce a primary wave followed by disaggregation; and high concentrations of ADP (10 mcg/mL final concentration) induce a single, broad wave of aggregation (Fig. 33-2). A biphasic aggregation response to ADP will not be seen in patients with platelet release disorders. Patients with Glanzmann's thrombasthenia show incomplete aggregation with ADP regardless of the final concentration.

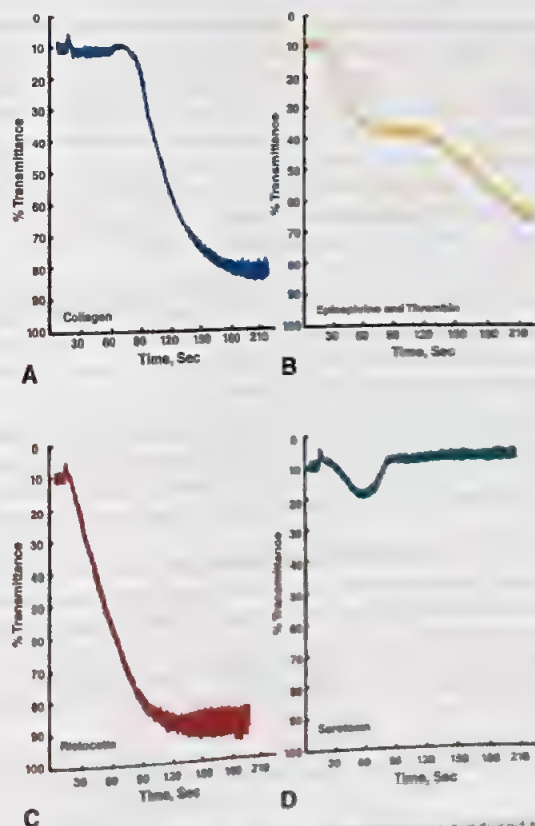


FIGURE 33-1 Aggregation curves with various aggregating agents. **A.** Aggregation curve induced by collagen. Note the lag time before aggregation followed by a single wave of aggregation. **B.** Aggregation curve induced with epinephrine and thrombin. Note the biphasic wave of aggregation. **C.** Aggregation curve induced by ristocetin. A single broad wave of aggregation may be seen. **D.** Aggregation curve induced by serotonin. Generally a single wave of aggregation followed by disaggregation is seen.

Continued

METHOD 33-3 Platelet Aggregation—cont'd

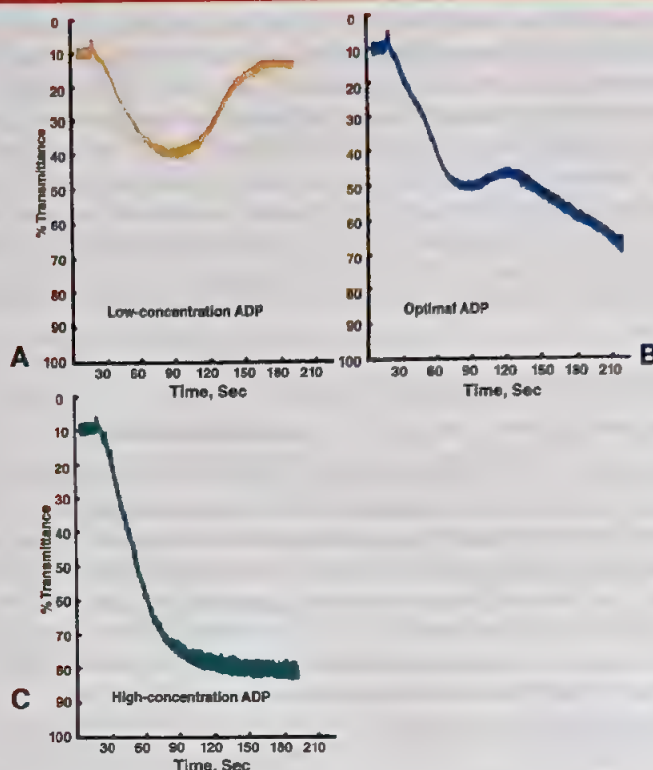


FIGURE 33-2 Aggregation curves induced with various concentrations of ADP. **A.** Very low concentrations of ADP induce a primary wave of aggregation followed by disaggregation. **B.** The optimal concentration of ADP induces a biphasic wave of aggregation. **C.** High concentrations of ADP induce a broad wave of aggregation.

Platelet aggregation induced by collagen is characterized by a lag period before aggregation, followed by only a single wave of aggregation. A biphasic aggregation response is seen with the antibiotic ristocetin; however, often only a single broad wave of aggregation will occur. Platelet aggregation induced with arachidonic acid causes a rapid secondary wave of aggregation. Biphasic aggregation is observed with epinephrine. One-third to one-half of normal, healthy patients produce only a primary wave of aggregation with epinephrine.⁸ The aggregating agent thrombin induces a biphasic wave of aggregation. Platelet aggregation induced by serotonin normally produces a primary wave of aggregation with a maximum of 10% to 30% transmittance followed by disaggregation⁸ (see Fig. 33-1).

In patients with severe von Willebrand's disease, aggregation to ristocetin is characteristically absent. Decreased to normal aggregation to ristocetin can be seen in patients with mild von Willebrand's disease. Correction of the abnormal ristocetin aggregation curves can be seen by the addition of normal, platelet-poor plasma to the patient's platelet-rich plasma. Abnormal ristocetin-induced platelet aggregation may also occur in patients with Bernard-Soulier syndrome, platelet storage pool defects, and idiopathic thrombocytopenia purpura (ITP).⁹

Limitations

There are some basic requirements for platelet aggregation as an *in vitro* means of evaluating platelet function:

- In performing platelet aggregation studies, a clean venipuncture is crucial. Hemolyzed samples should not be utilized because red blood cells contain ADP, which can prematurely activate platelets.
- Plasma from fasting patients is preferred for testing. Lipemic samples may obscure changes in optical density (OD) during platelet aggregation.
- Sodium citrate is the anticoagulant used in aggregation studies. Keep in mind that *in vitro* aggregation is dependent on the presence of calcium ions, and the mechanism of sodium citrate to prevent coagulation in the sample is by binding calcium ions. However, the concentration of calcium after anticoagulation with sodium citrate should still be sufficient for aggregation to occur.
- Fibrinogen must be present in the test sample for aggregation to occur.
- The plasma sample should not come in contact with a glass surface unless the surface is siliconized, because glass will cause platelet activation and adhesion to its surface.
- Aggregation studies should be performed at 37°C at a pH of 6.5 to 8.5. To help maintain pH values, all samples, once collected, should be capped to prevent CO₂ loss. The presence of CO₂ helps to maintain the pH of the sample.
- Test samples should be maintained at room temperature during processing. Cooling inhibits the platelet aggregating response. Just before performing the test, the plasma is incubated at 37°C in the heat block of the aggregometer.
- Platelet-rich plasma samples should be allowed to stand for approximately 30 minutes before performing aggregation testing. This is necessary for the platelets to regain their responsiveness after undergoing the preparation procedure.
- All aggregation studies should be performed within 3 hours of sample collection.

METHOD 33-3 Platelet Aggregation—cont'd

- Stirring is necessary to bring the platelets in close contact with one another to allow aggregation to occur.
- Agonists should be prepared fresh daily and brought to room temperature before use. They must have known potency and be added in small volumes.
- Control tests using platelet-rich plasma from a known normal donor must be performed at the same time as the patient samples.
- It is essential that a complete history be obtained from the patient, including all medications taken. They must refrain from taking any anti-inflammatory drugs and antiplatelet drugs for at least 1 week before testing. These drugs inhibit the platelets' release reaction.
- Thrombocytopenia makes evaluation of the aggregation responses difficult.

Reporting Results Primary and/or secondary aggregation or no response (absent)

Reference Ranges Collagen – single wave of aggregation
 Ristocetin – single broad wave of aggregation
 Arachidonic acid – causes a rapid secondary wave of aggregation
 Epinephrine – biphasic aggregation
 Thrombin – biphasic aggregation
 Serotonin – primary wave of aggregation

Notes In evaluating patients with suspected platelet disorders, the agonists most commonly used are ADP in varying concentrations, collagen, epinephrine, and ristocetin. Aspirin, aspirin compounds, and anti-inflammatory drugs inhibit the secondary wave of aggregation by inhibiting the release reaction of the platelet. Reduced or absent aggregation as well as disaggregation curves may be observed in patients taking medication containing aspirin. Other medications or substances have also been identified as inhibiting platelet function, such as ibuprofen, red wine, and a variety of herbs. Patients should be questioned carefully about possible ingestion of these substances before interpreting abnormal aggregation results.

The intensity of platelet aggregation may be estimated by recording the change in absorbance as a percentage of the difference in absorbance between platelet-rich and platelet-poor plasma. This has limited usefulness because absorbance is dependent on the size and density of platelet clumping and the number of platelets that aggregate. A more complex analysis of aggregation related to the rate of aggregation may also be obtained. However, visual interpretation of the aggregation curves suffices and can establish whether aggregation is abnormal or normal.

CRITICAL THINKING QUESTION

33-1 What are the potential limiting factors associated with the platelet aggregometry analysis?

See answers to all Critical Thinking Questions at the back of this book.

Coagulation Instrumentation

Methods to evaluate the coagulation of blood have evolved from the manual observation of the length of time needed for whole blood to clot in a test tube to today's intricate analysis performed using highly sophisticated instruments.

General Types of Coagulation Instrumentation**Fully Automated Method**

Fully automated analyzers became available within the past few decades and have greatly improved coagulation testing capabilities. All reagents are automatically pipetted into the test cuvette. Timers are automatically initiated and stopped when clot formation is detected. Plasma samples are automatically diluted and pipetted. Fully automated analyzers contain monitoring devices and internal mechanisms to maintain and monitor a constant 37°C temperature throughout the testing process. The advantage of these systems is their improved accuracy, precision, and throughput, with reduced time required for operator intervention.

Semiautomated Method

Semiautomated equipment is similar to the tilt-tube method in that it also requires that all reagents and test samples be delivered manually to the reaction cuvette. However, these instruments contain mechanisms to automatically initiate the timing device on addition of the final reagent and an internal mechanism to detect clot formation. The equipment usually contains a device for maintaining a constant 37°C temperature, but it may not internally monitor the temperature. The advantage of semiautomated equipment is that it is relatively inexpensive and easily operated. The disadvantage is that the testing process is time consuming and labor intensive.

Methods of Endpoint Detection

Instrument methodologies used for coagulation testing are generally classified based on the principle of endpoint detection employed by the analyzer. These general classifications are:

- Mechanical
- Photo-optical
- Chromogenic
- Immunological

Historically, coagulation instruments were only capable of providing one type of endpoint detection, such as mechanical or photo-optical. Photo-optical instruments were built to read

the endpoint at a fixed wavelength of 500 to 600 nm. Later, other instruments were developed to read at a 405-nm wavelength to allow measurement of chromogenic assays. Currently, several analyzers are available that have the combined capability of reading assays using multiple detection methods within the same system, allowing laboratories to purchase and train on only one instrument instead of multiple analyzers, while still providing specialized testing capabilities on all shifts with many operators.

Mechanical Endpoint Detection

Two primary methodologies are utilized for mechanical detection of clot formation. The first is known as electromechanical detection and incorporates a change in electrical conductivity between two metal probes immersed in a solution. During the reaction, one probe moves in and out of the solution at constant intervals. The electrical circuit between the two probes is not maintained as the moving probe rises in and out of the solution. When a clot (fibrin) is formed in the solution, the fibrin strands maintain electrical contact between the two probes when the moving probe leaves the solution, which stops the timer.¹⁰ An example of this type of equipment is the BBL Fibrometer®.

The second method of mechanical clot detection involves monitoring the movement of a steel ball within a test solution by magnetic sensors. A change in the movement of the steel ball may be detected when there is increased viscosity of the test solution, changing its range of motion, or by a break in contact with the magnetic sensors when the steel ball becomes incorporated into a fibrin clot as the cuvette rotates. These types of endpoint detection methods are utilized on the Diagnostica Stago STart analyzers¹¹ and the Trinity Biotech Destiny line.¹²

Photo-Optical Endpoint Detection

Detection of clot formation measured by a change in optical density (OD) of a test sample is the basis of photo-optical instrumentation, which is also known as turbidometric methodology. When a light source of a specified wavelength is passed through a test solution (plasma), a certain amount of light is detected by a photodetector or photocell located on the other side of the solution. The amount of light detected is dependent on the color and clarity of the plasma sample and is considered to be the baseline light transmission value. When soluble fibrinogen begins to polymerize into a fibrin clot, formation of fibrin strands causes light to scatter, allowing less light to fall on the photodetector (i.e., the plasma becomes more opaque, decreasing the amount of light detected). When the amount of light reaching the photodetector decreases to an exact point from the baseline value as predetermined by the instrument, this change in OD triggers the timer to stop, indicating clot formation. This is the most common method of detection utilized on coagulation instruments at this time, such as the MDAII® (Trinity Biotech),¹³ BCS® (Dade Behring), ACL TOP® analyzer (Instrumentation Laboratory), and the Destiny® (Trinity).

Chromogenic Endpoint Detection

Chromogenic, or amidolytic, methodology is based on the use of a specific color-producing substance known as a

chromophore. The chromophore normally used in the coagulation laboratory is *para*-nitroaniline (*p*-nitroaniline or pNA), which has an optical absorbance peak at 405 nm on a spectrophotometer. The principle of chromogenic assays is based on the attachment of pNA to a synthetic chromogenic substrate.¹⁴ The substrate is made up of a series of amino acids, the composition of which is dependent on the structure of the enzymatic target of the coagulation protein being measured. The goal is for the coagulation protein to attack the chromogenic substrate at a specific site between a defined amino acid sequence and the pNA, thereby cleaving pNA from the substrate. Because free pNA has a yellow color, the intensity of the solution is proportional to the amount of free pNA present and is measured by a photodetector at a wavelength of 405 nm. As additional free pNA is cleaved, the amount of light absorbance is increased, leading to a greater change in OD of the solution. The change in OD can be either a direct or an indirect measurement of the level of the analyte being tested. This type of technology is available on a variety of instruments such as the ACL TOP® Series (Instrumentation Laboratories), Sysmex CA600 series (Siemens Healthineers), and CoaLab 1000 (LABiTech Labor Biomedical Technologies) and is most often seen in conjunction with the photo-optical detection methodology.¹⁵

Immunological Endpoint Detection

Immunological assays are based on antigen-antibody reactions. Microlatex particles are coated with a specific antibody directed against the analyte (antigen) to be measured. A beam of monochromatic light is then passed through the suspension of microlatex particles. When the wavelength of light is greater than the diameter of the particles in suspension, only a small amount of light will be absorbed by the particles. When the microlatex particles coated with specific antibody come in contact with the antigen present in the solution, the antigen attaches to the antibody and forms bridges between the particles, causing them to agglutinate. As the diameter of the agglutinates becomes larger and closer to the wavelength of the monochromatic light beam, the greater the amount of light that is absorbed. The increase in light absorbance is proportional to the size of the agglutinates, which, in turn, is proportional to the antigen level present in the sample, which is read from a standard curve. This technology is also incorporated into several of the instruments previously mentioned.

The introduction of new methodologies has provided the ability to perform new assays in the coagulation laboratory. Refinement of these methods has increased our ability to recognize and improve our diagnostic capabilities for determining the causes of disorders of hemostasis and thrombosis.

Complete Hemostasis Assessment

Thromboelastography (TEG)

Thromboelastography (TEG) is an assay used to determine clotting factor activation, rate of clot formation, clot strength, and fibrinolysis on a point-of-care platform. Analysis requires approximately 350 μ L of whole blood. The sample is

incubated at 37°C in a cylindrical cup. A pin on a torsion wire is suspended in the sample and the cup containing the blood sample is rotated 4°45' every 10 seconds to simulate venous flow. Initially the pin does not move as the cup rotates, but as the viscoelastic strength increases with the formation of the clot, the pin becomes caught in the fibrin and moves. The oscillation of the pin is detected through the torsion wire by an electromagnetic transducer.

The initial clotting time is assessed from the point that an activator is added to when the clot forms. Clot formation is defined as 2 mm in amplitude on computer reading. Initial clotting can take up to 5 to 10 minutes and is an assessment of factors or anticoagulants in the blood sample. The strength of the clot is measured from the initial clot at 2 mm amplitude until 20 mm amplitude is detected. This step takes from 1 to 3 minutes and is associated with fibrinogen. The rate of clot formation is a computation between the time of the initial clot to the 20 mm amplitude clot measurement. This value is also associated with fibrinogen. Abnormalities in either stage may indicate the need to transfuse fibrinogen rich cryoprecipitate. The maximum amplitude measured is proportional to maximum clot strength. Platelets as well as fibrin strands and factors II and III contribute to maximum clot strength. The normal maximum strength ranges from 55 to 75 mm in amplitude. Abnormalities in this stage may indicate a need to give platelets or DDAVP. Finally, clot stability is the percent of the clot remaining 30 minutes following the maximum clot strength. This parameter determines the fibrinolytic pathway and the need for antifibrinolytic treatments. The normal value is 0% to 8% (Figure 33-3).

Clinical applications of thromboelastography provide more detailed information allowing for informed decisions on treatments. TEG is useful as an initial screening assessment for

clotting disorders that are especially important in cardiac and liver surgeries as well as with trauma cases.¹⁶ Knowledge of potential bleeding issues can alert physicians of complications and reduce blood product use. A more complete assessment of coagulation in a bleeding patient allows the use of the most effective blood components. Reduced sample size for coagulation testing is also beneficial in pediatric patients. Finally, factor deficiencies are often heterogeneous in coagulation severity. TEG provides a full evaluation of clotting from initiation to lysis and reveals better treatment options. TEG is often used as a screening test since additional information is required before patient treatment.¹⁷

Rotational Thromboelastometry (ROTEM)

An alternative to TEG is rotational thromboelastometry (ROTEM). There are many similarities between the two assays. The ROTEM system uses a fixed cylindrical cup with a pin suspended on a ball bearing that rotates at 4°75' every 6 seconds. As the clot strengthens the pin moves with the rotation and is detected by an optical sensor. Each method provides insight into clotting time, rate, maximum strength, and fibrinolysis, although different nomenclature is used. Since the methodologies differ, the results cannot be interchanged.

Coagulation Screening Tests

Method 33-4: Activated Partial Thromboplastin Time

The activated partial thromboplastin time (aPTT) is a screening test used to evaluate the intrinsic and common pathway of coagulation, or more precisely, to measure all the plasma coagulation factors with the exception of factors VII and XIII (Fig. 33-4).

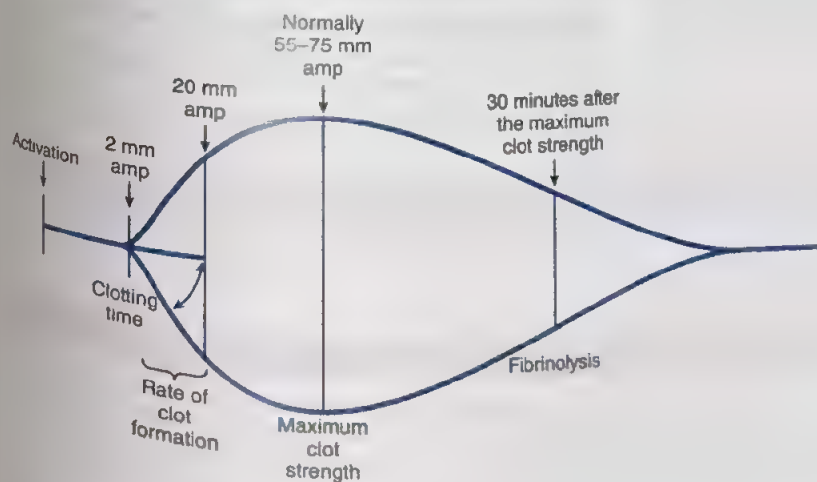


FIGURE 33-3 Normal thromboelastography pattern.

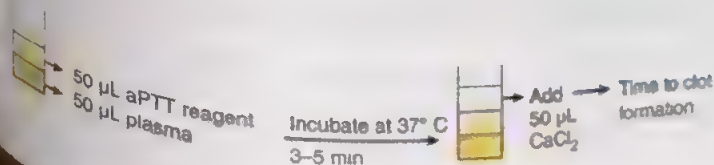


FIGURE 33-4 Activated partial thromboplastin time (aPTT) assay.

METHOD 33-4 Activated Partial Thromboplastin Time

Principle	The formation of fibrin occurs at a normal rate only if the factors involved in the intrinsic pathway (factors VIII, IX, XI, and XII) and the common pathway (factors I, II, V, and X) are present in normal concentrations with normal functionality. Optimal activation is achieved by the addition of contact activators such as kaolin, celite, micronized silica, and ellagic acid, which eliminate the variability of activation by glass contact. The aPTT reagent also includes a platelet phospholipid substitute, which eliminates the test's sensitivity to platelet number and function. Once the aPTT reagent is added, the factors are activated and CaCl_2 (0.02 M) is added. The time to clot formation after the addition of calcium is determined. The aPTT is also used to screen for inhibitors of the intrinsic pathway, such as the lupus anticoagulant, and to monitor heparin therapy. ¹⁸
Specimen	Sodium citrate anticoagulated plasma
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	Each laboratory should develop its own normal range based on the type of analyzer, reagent, and patient population. The test result may be prolonged in patients with deficiencies of any factor involved in the intrinsic or common pathway. The aPTT may be prolonged when factor levels are less than 30% to 40% of normal, depending on reagent sensitivity. Hypofibrinogenemia (levels less than 100 mg/dL) will also prolong the aPTT.
Limitations	Because commercial aPTT reagents vary in the type of activator used, they will have different requirements for optimal contact activation time between the reagent and plasma during the testing process. The manufacturer's recommendations should be followed and the incubation time on the coagulation analyzer set accordingly. Failure to adhere to correct incubation times for the specific reagent used may yield erroneous results.
Reporting Results	aPTT is reported in seconds
Reference Ranges	Reference ranges are set per institution but vary around 25 to 35 seconds
Notes	Both the aPTT and the prothrombin time (PT) should be performed when screening samples for coagulopathies, because together they evaluate the intrinsic, extrinsic, and common pathways of coagulation.

Method 33-5: One-Stage Prothrombin Time (Quick)

The **prothrombin time (PT)** is a valuable screening procedure used to indicate possible factor deficiencies of the extrinsic or common pathway.¹⁹ The PT test is sensitive to the vitamin K–dependent factors of the extrinsic (factor VII) and common pathways (factors II and X) and is, therefore, used as a means of monitoring oral anticoagulant therapy (Fig. 33-5). *Note:* The fourth vitamin K–dependent procoagulant, factor IX, is measured by the aPTT.



FIGURE 33-5 Prothrombin time (PT) assay.

Method 33-6: Thrombin Time

The **thrombin time (TT)** is the time required for thrombin to convert fibrinogen to an insoluble fibrin clot. Bovine thrombin is most commonly used, but human thrombin may be used and is beneficial, especially for patients who may have an antbovine thrombin antibody, which occurs in some patients exposed to bovine topical thrombin.

Method 33-7: Mixing Studies—aPTT or PT 1:1 Mix

A patient with an abnormal PT and/or aPTT with no known cause is a good candidate for mixing studies. A mixing study involves mixing the patient's plasma 1:1 with normal plasma and repeating the test in question. Mixing studies producing results closer to normal are indicative of factor deficiencies whereas results that do not correct with normal plasma are more indicative of coagulation inhibitors.

METHOD 33-5 One-Stage Prothrombin Time (Quick)

Principle	The PT is a measure of the extrinsic and common pathways of coagulation involving factors II, V, VII, and X (as well as fibrinogen). Therefore, the time required for the formation of a fibrin clot when plasma is added to a thromboplastin–calcium mixture is reported as the Prothrombin Time. Tissue thromboplastin is added to activate factor (F) VII which activates FV to FVa, that participates in formation of the prothrombinase complex (FXa + FVa on a phospholipid surface), ultimately generating thrombin from prothrombin (factor II). The thrombin thus formed converts fibrinogen to fibrin. The rate of fibrin formation depends on the level of factors II, V, VII, and X and fibrinogen. Therefore, the PT can be used as an indicator of the overall activity of these factors.
Specimen	Sodium citrate anticoagulated plasma
Procedure	See the required equipment and steps for this procedure at www.fadavis.com

METHOD 33-1 One-Stage Prothrombin Time (Quick)—cont'd

Interpretation	The PT is prolonged in individuals with a factor deficiency involving a single factor (i.e., patients with a congenital deficiency) or involving multiple factors (i.e., patients with acquired deficiencies such as liver disease, oral anticoagulant therapy, or patients with vitamin K deficiency). The PT may also be prolonged in the presence of FDPs and heparin, depending on the reagent used.
Limitations	In patients with polycythemia, the PT is prolonged as a result of a change in the ratio of anticoagulant to plasma. For coagulation testing, the blood to sodium citrate ratio should always be maintained at 9:1. Elevated hematocrits greater than 55% (such as in polycythemia) yield a smaller amount of plasma in the collection tube, thereby increasing the proportion of anticoagulant in the plasma. Increased citrate will bind the calcium chloride added to the system during the testing process, causing a prolongation of the PT. In this case, the amount of citrate should be adjusted (decreased) before specimen collection to compensate for the elevated hematocrit, as previously described. PT results may be shortened when the plasma is stored for longer than 4 hours at 4°C because of cold activation of factor VII.
Formulas	$\text{INR} = \left\{ \frac{\text{Patient PT}}{\text{Geometric mean of normal range}} \right\}^{2.0}$
Reporting Results	PT results are reported in seconds
Reference Ranges	11 to 13 seconds
Notes	The reference range for PT is approximately 10 to 13 seconds. This range varies with the type of thromboplastin employed in the testing process and the method of clot detection. Each laboratory must develop its own reference range based on the type of analyzer, reagent, and patient population.

METHOD 33-6 Thrombin Time

Principle	Reagent thrombin is added to the specimen that directly converts fibrinogen to fibrin.
Specimen	Sodium citrate anticoagulated plasma
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	The TT is prolonged in patients with hypofibrinogenemia (usually less than 100 mg/dL), dysfibrinogenemia, and in the presence of anticoagulants such as heparin and hirudin, or FDPs. If the 1:1 mixing study results in a clotting time that approximates that of the control plasma, a deficiency or a molecular abnormality of fibrinogen is most likely indicated. If the mixing study fails to correct the TT, the presence of an inhibitor such as heparin is indicated.
Limitations	The TT is affected by abnormal levels of fibrinogen and dysfibrinogenemia and the presence of antithrombins such as heparin and direct thrombin inhibitors such as hirudin and FDPs.
Reporting Results	The thrombin time is reported in seconds
Reference Ranges	15 to 22 seconds
Notes	The reference value depends on the particular reagent and instrument system used.

METHOD 33-7 Mixing Studies: aPTT or PT 1:1 Mix

Principle	Mixing studies using the aPTT and/or PT are useful screening tests for distinguishing between factor deficiencies and coagulation inhibitors. Patient plasma mixed with pooled normal plasma (with approximately 100% factor levels) should contain sufficient factor levels (at least 50% normal level) to correct a prolongation caused by a factor deficiency. An inhibitor is most likely present if there is only partial or no correction in the PT and/or aPTT when mixed 1:1 with normal plasma. Mixing studies can help determine the appropriate next steps to take to diagnose the cause of an abnormal aPTT or PT.
Specimen	Sodium citrate anticoagulated plasma
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	<p>If the aPTT or PT is corrected (or normalized) by normal plasma, a factor deficiency is indicated. The addition of normal plasma supplies the coagulation factor or factors that are deficient, and thus corrects the aPTT or PT.</p> <p>If the aPTT or PT is not corrected by the addition of normal plasma immediately, a strong inhibitor is indicated.²⁸</p> <p>A weak or time-dependent inhibitor is indicated by a prolonged aPTT or PT following incubation at 37°C for 1 to 2 hours. A weak inhibitor progressively inactivates the coagulation factor, thus prolonging the aPTT or PT. This time-dependent pattern is most typical of a factor VIII inhibitor.</p>

Continued

METHOD 33-7 Mixing Studies—aPTT or PT 1:1 Mix—cont'd

Limitations	If a factor VIII inhibitor is present, it is important to determine the initial level of factor activity because the development of an inhibitor complicates the management of a patient with Hemophilia A when therapy involves AHF concentrates. These should be monitored periodically.
Reporting Results	The PT and/or aPTT performed in mixing studies is reported in seconds and alongside the undiluted sample results.
Reference Ranges	See previously provided reference ranges for the PT and/or aPTT.
Notes	The antibody that inhibits factor VIII is most often a specific IgG antibody developed through an autoimmune disorder. ¹ These antibodies are often present as weak inhibitors but are temperature and time dependent, thus causing only a slightly prolonged aPTT on initial testing. Mixing tests may yield aPTT results intermediate between the clotting times of patient and normal control. On incubation at 37°C, both the undiluted patient plasma and plasma mixtures show prolonged times, but the normal pooled plasma shows little or no change. Because of the nature of the factor VIII inhibitor, the mixture of patient plasma and normal pooled plasma must be incubated for 60 to 120 minutes to allow for the inhibitor's progressive activity.

Coagulation Factor Assays**Method 33-8: One-Stage Quantitative Assay Method for Factors II, V, VII, and X**

The prothrombin time (PT) is the basis of this test system, with specific factor-deficient plasmas being added to the patient plasma. The percentage of factor activity is determined by the amount of correction of the PT when specific dilutions of patient plasma are added to the factor-deficient plasma. These results are obtained from an activity curve made using clotting times of dilutions of normal reference plasma and specific factor-deficient plasma.

Method 33-9: One-Stage Quantitative Assay Method for Factors VIII, IX, XI, and XII

The activated partial thromboplastin time (aPTT) is the basis of this test system, with specific factor-deficient plasmas being added to the patient plasma. The percentage of factor activity is determined by the amount of correction of the aPTT when specific dilutions of patient plasma are added to

the factor-deficient plasma. These results are obtained from an activity curve made using clotting times of dilutions of normal reference plasma and specific factor-deficient plasma.

Method 33-10: Factor XIII Chromogenic Assay (Activity)

Deficiency in factor XIII is very rare, and since it is not involved in the formation of the initial unstable clot, it is not detected in PT or aPTT tests. When a patient is bleeding, has poor wound healing, and coagulation testing (PT, aPTT, TT, and Fibrinogen) is normal, a deficiency in factor XIII can be considered.

Coagulation Inhibitors

The circulating antibody directed against the factor VIII molecule is the most common specific factor inhibitor. It is seen in patients with hemophilia A and may be related to repeated therapeutic transfusions of antihemophilic factor (AHF) but is also seen in nonhemophiliac patients (e.g., women after childbirth or abortion, elderly individuals, and those with

METHOD 33-8 One-Stage Quantitative Assay Method for Factors II, V, VII, and X

Principle	Normal reference plasma with 100% factor activity is diluted. Factor-deficient plasma is added in each reference plasma concentration to establish a reference curve for factor activity quantitation. A PT is performed on patient plasma mixed with factor deficient samples.
Specimen	Sodium citrate anticoagulated blood
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	PT results that are within reference limits indicate that the factor present in the patient plasma corrected the reagent deficient plasma. PT results that are abnormal indicate that both the reagent deficient plasma and patient plasma are deficient in the corresponding factor.
Limitations	Inhibitors will often have a "dilutional" effect, demonstrating nonparallel curves with increasing dilutions. This should be considered if the results of the 1:10, 1:20, and 1:40 dilutions do not agree within 15%. In this case, results should not be averaged, but further dilutions such as 1:80 and 1:160 performed until results of two consecutive dilutions match within 15% and measure within linearity of the calibration curve.
Reporting Results	Factor activity is reported in %
Reference Range	An approximate range of 50% to 150% is considered normal.
Notes	Each laboratory should define its own reference range based on instrument, reagent, and patient population.

METHOD 33-9 One-Stage Quantitative Assay Method for Factors VII, IX, XI, and XII

Principle	This method is based on the ability of patient plasma to correct specific factor deficient plasma as determined by the aPTT test. Results in percent activity are obtained from an activity curve.
Specimen	Sodium citrate anticoagulated plasma
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	<ul style="list-style-type: none"> If the result from the 1:20 dilution is greater than the highest curve point (i.e., 1:10), prepare additional dilutions of the patient sample with buffered saline until results fall within the linearity range of the curve. If the result from the 1:10 dilution is lower than the lowest curve point (i.e., 1:1,280), report the factor activity as less than the lowest calibrator value. To calculate the percent activity for additional dilutions tested, multiply the measured result by the dilution ratio of the sample to the 1:10 dilution (e.g., 1:40/1:10 = dilution factor of 4) to determine the percent activity of the patient sample. These tests require the same considerations as the aPTT and PT assay in regard to quality control, specimen handling, reagent preparation, and points of procedural importance.
Limitations	<p>Inhibitors will often have a "dilutional" effect demonstrating nonparallel curves with increasing dilutions. This should be considered if the results of the 1:10, 1:20, and 1:40 dilutions do not agree within 15%. In this case, results should <i>not</i> be averaged, but further dilutions such as 1:80 and 1:160 performed until two consecutive dilutions match each other within 15% and are within the measurable linearity of the curve.</p> <p>Read the percent activity directly from the activity curve (similar to that displayed in Fig. 33-6). If using an automated analyzer, the results may be automatically read from the curve and printed out.</p> <p><i>Note:</i> Specific volumes required for adding factor-deficient plasma, diluted patient plasma, and aPTT reagent may vary depending on the automated analyzer used. The volumes previously recommended may be used if performing the test manually.</p>

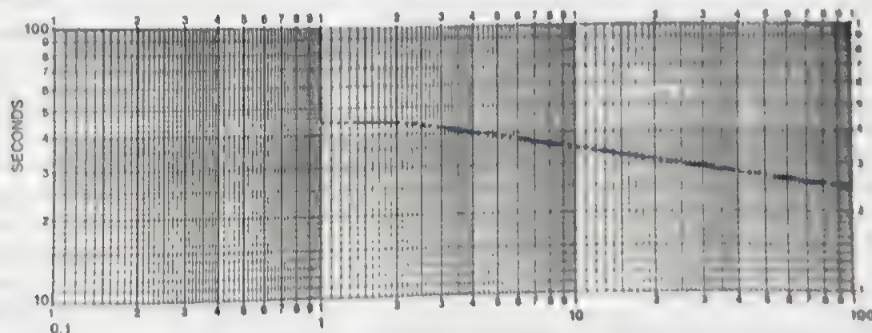


FIGURE 33-6 Factor V activity curve.

Reporting Results	Factor activity is reported in %
Reference Range	An approximate range of 50% to 150% is considered normal.
Notes	Each laboratory must define its own normal range based on instrument, reagent, and patient population.

METHOD 33-10 Factor XIII Chromogenic Assay (Activity)

Principle	Fibrin produced by the action of thrombin is prevented from forming a clot by the action of a polymerase inhibitor (Gly Pro Arg-Pro Ala-amide). When FXIII is activated by thrombin, FXIIIa cross links a specific peptide substrate to glycine ethyl ester, which releases ammonia. The amount of ammonia released is monitored by a glutamate dehydrogenase catalyzed reaction, which releases ammonia. The amount of NADH is measured spectrophotometrically by the decreased absorbance at 340 nm.
Specimen	Sodium citrate anticoagulated plasma
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	Results that are within reference ranges indicate that factor XIII is functioning properly. A deficiency in FXIII will result in low test results.
Limitations	False-negative results can be caused by a cloudy or lipemic plasma sample. False-positive results can be caused by increased bilirubin in plasma.

METHOD 33-10 Factor XIII Chromogenic Assay (Activity)—cont'd

Reporting Results	Factor activity is reported as %
Reference Ranges	An approximate range of 57% to 192% is considered normal.
Notes	Each laboratory must define its own normal range based on instrument, reagent, and patient population.

immunological disorders such as rheumatoid arthritis). This is usually attributed to the elevated level of factor VIII present, which is interpreted by the immune system as being foreign. Antibodies to factor VIII may also be seen in patients known to have the severe form of von Willebrand's disease.

Other factor-specific inhibitors have been reported against factors V, IX, XI, XII, and XIII. Some patients with a tendency to thrombose develop an acquired circulating inhibitor. This inhibitor is known as the lupus anticoagulant, as it was first seen in patients with systemic lupus erythematosus, and it demonstrates activity against phospholipid-protein complexes, thus interfering with phospholipid-dependent complexes that involve factors V and VIII.²²

Factor VIII inhibitors may be quantified by mixing the patient plasma with pooled plasma containing a known amount of factor VIII. The mixture is incubated at 37°C for 2 hours. The amount of inhibitor present is then calculated in Bethesda units by comparing the difference in factor activity between the patient mixture and a control mixture containing the pooled plasma and buffer. A Bethesda unit of inhibitor activity is defined as the amount of inhibitor that will inactivate half of the factor present in a mixture of equal volumes of patient plasma and pooled plasma after 2 hours of incubation at 37°C. The definition was originally defined to measure factor VIII inhibitors; however, the same method can be used to define a specific inhibitor against any factor.

If a factor VIII inhibitor is present, it is important to determine the initial level of factor activity, because the development of an inhibitor complicates the management of a patient with hemophilia A when therapy involves AHF. Evaluation of the level of inhibitor is necessary to make decisions regarding treatment. Therapeutic options for patients with low titer factor VIII inhibitors are often treated with higher doses of factor replacement during bleeding episodes. Desensitization and immunosuppression are commonly used with patients exhibiting higher titers of inhibitors. Additionally, therapeutic options using bypass agents (recombinant FVIIa; Novoseven RT, Novo Nordisk, and activated prothrombin complex concentrate; FEIBA VH, Baxalta) are frequently used to trigger an alternate route for thrombin formation in patients with inhibitors.²³

CRITICAL THINKING QUESTION

33-2 How does testing progress from screening to diagnosis when a patient is being assessed for a factor deficiency?

Tests to Monitor Anticoagulant Therapy**Monitoring Anticoagulant Therapy With Coagulation Screening Assays****Heparin Therapy**

When the aPTT is used to monitor heparin therapy, a heparin therapeutic range should be determined for the reagent/instrument system in use by comparing the aPTT to the antifactor Xa heparin activity in at least 30 patient samples. The samples selected for the heparin therapeutic range study should span the measurable range of the aPTT assay, with no more than two samples from any one patient. The patient should not be on concomitant warfarin therapy. The aPTT is plotted versus the heparin anti-Xa assay result and the linear regression equation calculated. The aPTT range that correlates to 0.3 to 0.7 U/mL heparin activity is the recommended therapeutic range for unfractionated heparin. Although more expensive, the anti-Xa assay is preferred for accurate unfractionated heparin dosing.¹⁸

Anti-Vitamin K (Warfarin) Therapy

The PT is the most commonly ordered coagulation assay, largely owing to its use when monitoring warfarin therapy. Warfarin blocks gamma-carboxylation of the vitamin K-dependent coagulation factors and inhibitors in the liver—factors II, VII, IX, X, protein S, and protein C. The result is formation of inactive forms of the proteins. The PT is prolonged owing to the decrease in factor activity, and patients are commonly monitored with the calculated INR. The therapeutic range for most indications is an INR of 2.0 to 3.0, with a higher range used in patients with mechanical heart valves and those resistant to warfarin.

Method 33-11: Anti-FXa Assay (Heparin Activity)

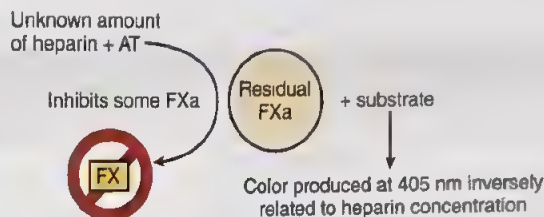
Although the factor Xa-assay and the aPTT are both used to evaluate heparin therapy, they differ in clotting assessment. Heparin enhances antithrombin III activity to prevent clotting where antithrombin III binds and inactivates FIXa, FX, FXIa, FXIIa, and thrombin. The aPTT measures both the intrinsic and common pathways; however, the factor Xa-assay measures the common pathway alone.

Monitoring Direct Thrombin Inhibitors

Several agents are in clinical use that anticoagulate by directly inhibiting thrombin and blocking its ability to convert fibrinogen to fibrin. Varieties of hirudin, an anticoagulant first discovered in leeches, and now manufactured with recombinant methods, include lepirudin, desirudin, and bivalirudin. Argatroban as well as dabigatran are synthetically manufactured. The PT is too responsive to these agents; aPTT response flattens out in the critical measurement range, and the thrombin time is frequently prolonged over the measurable range of the

METHOD 33-11 Anti-FXa Assay (Heparin Activity)**Principle**

Plasma containing unfractionated, low molecular weight, or direct antifactor Xa (anti-FXa) inhibitors is added to a reagent containing a constant amount of FXa. In the presence of antithrombin, either added as a separate reagent or present in the patient's plasma, inhibition of FXa will occur in proportion to the amount of heparin present. A chromogenic substrate is added and any residual active FXa will cleave the substrate, producing a color change. The optical density is monitored at 405 nm, with the degree of color produced inversely proportional to the amount of heparin in the sample (Fig. 33-7). This procedure is easily automated on most current coagulation analyzers.

**FIGURE 33-7** Heparin anti-FXa assay.

Specimen	Sodium citrate anticoagulated plasma
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	Therapeutic levels: Unfractionated heparin = 0.3 to 0.7 IU/mL Low molecular weight heparin = 0.6 – 1.1 IU/mL if dosed twice per day; 1.0 to 2.0 IU/mL if dosed once per day
Limitations	The calibration curve should be prepared from the same type of anticoagulant the patient is on, i.e., unfractionated heparin, low molecular weight heparin (Lovenox®, Fragmin® for example), or fondaparinux (Arixtra®).
Reporting Results	Reported in IU/mL
Reference Range	Reference ranges are established in each facility based in the reagents, lot numbers, and anticoagulant of interest
Notes	Anti-FXa assays should never be performed if the type of therapeutic heparin is unknown. Assays must be specific to therapies.

assay when these agents are present, so alternative assays are necessary that have a responsive, measurable range inclusive of the therapeutic ranges of these drugs. Assays that have this ability, though not in common laboratory use, include the ecarin clotting time and the Prothrombinase-induced clotting time (PiCT).

Tests to Measure Fibrin Formation**Method 33-12: Reptilase Time**

The reptilase time is useful in determining whether thrombin inhibitors, such as heparin, are causing a prolonged TT. The snake venom enzyme, reptilase, is resistant to the effects of heparin.

METHOD 33-12 Reptilase Time**Principle**

The reptilase time is similar to the TT except that the clotting sequence is initiated with the snake venom enzyme reptilase. Reptilase is thrombin-like in nature and hydrolyses fibrinopeptide A from the intact fibrinogen molecule. This is in contrast to thrombin, which hydrolyses fibrinopeptide A and B from fibrinogen. The clot that forms as the result of the action of reptilase on fibrinogen is more fragile than that formed by the action of thrombin on fibrinogen. The advantage of reptilase time is that it is not inhibited by heparin. There is only a minimal effect on the reptilase time by FDPs.

Specimen

Sodium citrate anticoagulated plasma

Procedure

See the required equipment and steps for this procedure at www.fadavis.com

Interpretation

In the presence of heparin, thrombin is inhibited through the interaction of antithrombin (AT-III). However, heparin does not interfere with the ability of reptilase to cleave fibrinopeptide A from fibrinogen. A comparison of both TT and reptilase time will aid in detecting the presence of thrombin inhibitors such as heparin.

Limitations

Infinite reptilase times are seen with fibrinogen_{deficiency} and all congenital dysfibrinogenemias. The reptilase time is also infinitely prolonged in cases of congenital afibrinogenemia. In states of hypofibrinogenemia, the reptilase time may be variable, depending on the levels of fibrinogen present. The reptilase time is moderately prolonged in the presence of FDPs and is unaffected by heparin. Table 33-2 compares thrombin time with reptilase time and identifies related defects.

Continued

METHOD 33-12 Reptilase Time—cont'd

Reporting Results	Reported in seconds
Reference Ranges	18 to 22 seconds
Notes	The reptilase test can be used to confirm heparin contamination and thrombin clotting time results in factor VIII deficiency and heparinized samples. ²⁴

TABLE 33-2 Comparison of Thrombin and Reptilase Time and Associated Defects

Thrombin Time	Reptilase Time	Defect
Infinitely prolonged	Infinitely prolonged	Dysfibrinogenemia
Infinitely prolonged	Infinitely prolonged	Afibrinogenemia
Prolonged	Equally prolonged	Hypofibrinogenemia
Prolonged	Normal	Heparin
Prolonged	Slightly to moderately prolonged	FDPs

Method 33-13: Fibrinogen Activity

Fibrinogen activity testing is necessary to identify the quantity of patient fibrinogen after an abnormal TT result.

Tests for von Willebrand's Disease

Von Willebrand's disease (vWD) is seen in approximately 1 in 10,000 symptomatic patients, making it one of the most

common inherited bleeding disorders. The **von Willebrand factor (vWF)** molecule is multifunctional in hemostasis acting in platelet-subendothelium and platelet-to-platelet adhesion as well as in platelet aggregation. The vWF molecule has specific binding sites for factor VIII, glycoprotein (GP) Ib, and GPIIb/IIIa platelet receptors, collagen, sulfatides, heparin, and snake venom-derived botrocetin. It interacts with the GP Iba subunit platelet receptors, functioning to anchor platelets to an

METHOD 33-13 Fibrinogen Activity

Principle	Fibrinogen can be quantitatively measured by a modification of the TT because the thrombin clotting time of dilute plasma is inversely proportional to the concentration of fibrinogen. This method involves testing dilutions of both patient plasma and control plasma with an excess of thrombin and is referred to as the Clauss method. Results are calculated from a calibration curve (see Fig. 33-8).
Specimen	Sodium citrate anticoagulated plasma
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	<p>Prolonged clotting times may indicate either a low fibrinogen concentration or the presence of inhibitors such as heparin or circulating FDPs.</p> <p>A comparison of clotting times using both TT and reptilase time may help to distinguish a fibrinogen deficiency from a dysfibrinogenemia.</p> <p>Low fibrinogen activity is seen in infants and children and in those with congenital afibrinogenemia, dysfibrinogenemia, or hypofibrinogenemia. Acquired deficiencies are seen in liver disease, disseminated intravascular coagulation (DIC), and fibrinolysis.</p> <p>High fibrinogen levels are seen during pregnancy and in women taking oral contraceptives. Fibrinogen is considered an acute-phase reactant, and, therefore, high levels may be seen in states of acute infection, neoplasms, collagen disorders, nephrosis, and hepatitis along with other conditions causing physical stress.</p>
Limitations	If a prolonged clotting time is obtained using a 1:10 dilution of patient plasma, this may indicate low fibrinogen levels of 50 mg/dL or less. Retest the sample using a 1:5 or 1:2 dilution with buffered saline and divide the results by 2 or 5, respectively, to obtain the final result. If a short clotting time is obtained using a 1:10 dilution of patient plasma, this may indicate high fibrinogen levels of 400 mg/dL or more. Retest the sample using a 1:20 dilution and multiply the results by 2 to obtain the final results.

METHOD 33-18 Fibrinogen Activity—cont'd**Reporting Results**

Reported in mg/dL

Reference Ranges

200 to 400 mg/dL

Notes

A number of automated instruments are available that measure fibrinogen concentrations based on change in OD as part of the PT determination. This is known as a PT-derived fibrinogen. Fibrinogen activity determined by this methodology is more susceptible to interfering substances such as increased levels of FDPs, which may cause unreliable results.

Fibrinogen antigen levels may also be assayed by means of radial immunodiffusion (RID) or nephelometry. This is a measure of the total amount of fibrinogen protein present, compared with fibrinogen activity, which is a measure of its functional ability. Determination of both fibrinogen antigen and activity levels is useful in evaluating dysfibrinogenemia, which will show abnormal fibrinogen activity with normal antigen levels.

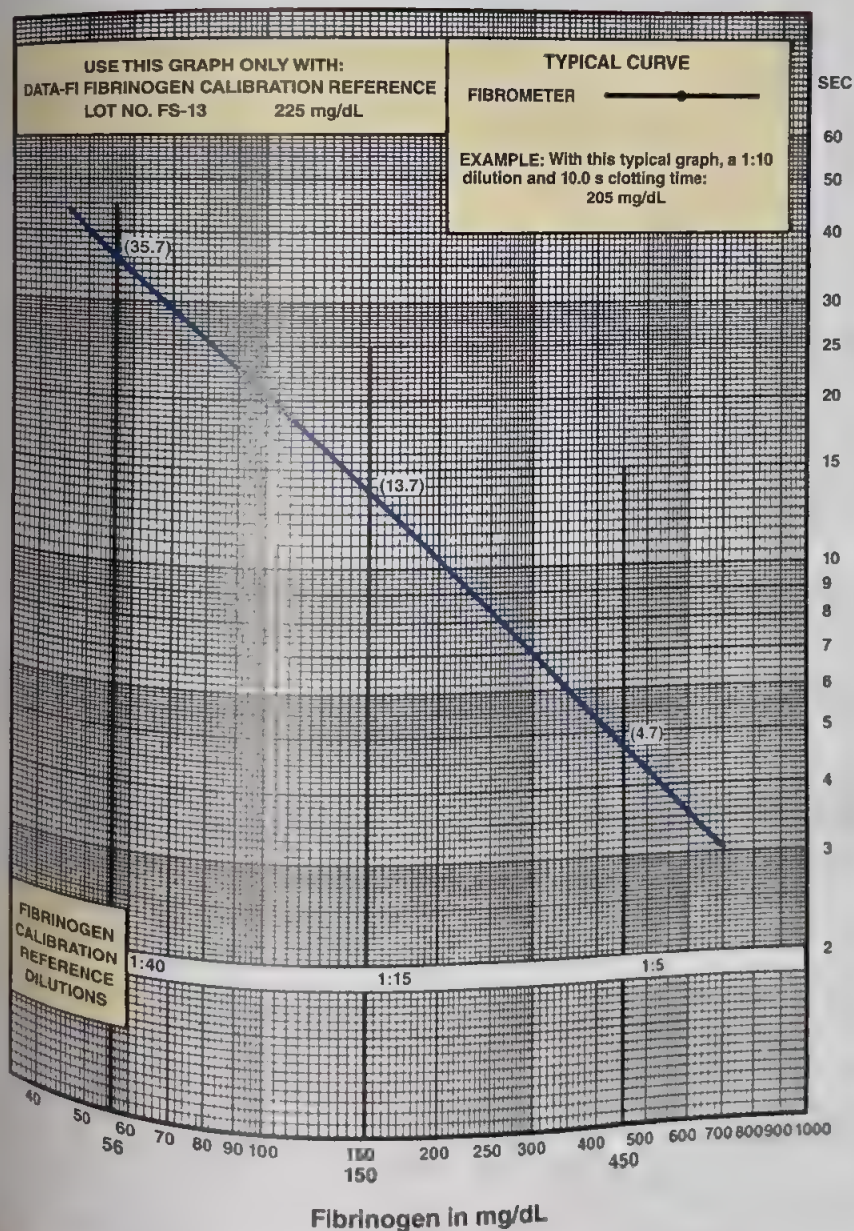


FIGURE 33-8 Fibrinogen calibration curve

exposed collagen surface. vWF has a very high binding affinity for factor VIII and thus acts as a carrier molecule to extend the half-life of factor VIII in circulation by protecting it from proteolytic degradation from circulating proteases.²⁵

The vWF molecule forms dimers within itself that range in size from 500 to 20,000 kDaltons. The high molecular weight multimers are responsible for most of the hemostatic activity as determined by ristocetin cofactor activity or collagen binding assays. ADAMTS-13 is a circulating protease that cleaves large vWF multimers at the subendothelium to regulate thrombi development. Deficiencies in ADAMTS-13 result in thrombotic excesses.²⁶ vWF is synthesized by megakaryocytes and in endothelial cells and is also present in the α -granules or specific Weibel-Palade bodies of platelets. von Willebrand's disease is categorized into types based on the presence or absence of high molecular weight multimers (Table 33-3).

Method 33-14: von Willebrand Factor Antigen

Von Willebrand factor antigen (vWF:Ag) can be quantitated using **enzyme-linked immunosorbent assay (ELISA)**, **Lau-rell rocket electrophoresis**, or **latex immunoassay (LIA)**. These methods measure the total von Willebrand factor protein independent of its ability to function. The automated LIA method and the ELISA method, using the sandwich technique, are most commonly used for the quantitative determination of vWF:Ag (Figs. 33-9 and 33-10).

In the LIA method, microlatex beads are coated with specific antihuman vWF antibodies. When plasma is added, the vWF present in the sample binds to the latex beads, causing aggregation of the microparticles. The optical density (OD) is monitored at 570 nm, and the quantity of vWF in the sample is directly related to the increased OD of the solution. This method is commonly automated on coagulation analyzers.

Method 33-15: von Willebrand Factor Activity (vWF:RCO, Ristocetin Cofactor)

In von Willebrand's disease, the amount of agglutination is usually decreased, with the exception of type 2N and "pseudo" or "platelet-type" von Willebrand deficiency. Levels of vWF

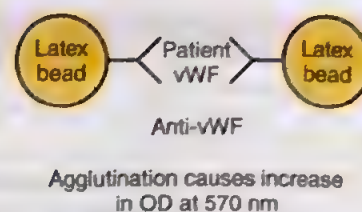


FIGURE 33-9 Latex immunoassay principle.

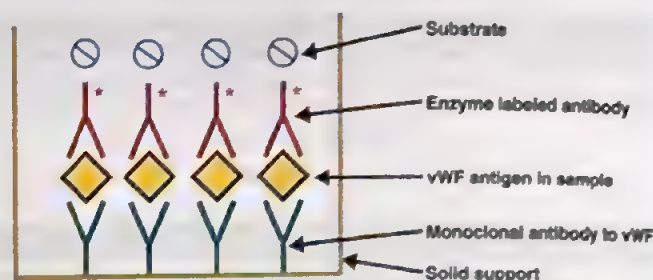


FIGURE 33-10 Principle of the sandwich enzyme-linked immunosorbent assay (ELISA) test for von Willebrand factor antigen (vWF:Ag). (Modified from Constantine, N, et al. *Retroviral Testing, Essentials for Quality Control and Laboratory Diagnosis*. Boca Raton, FL: CRC Press; 1992, p. 71, with permission.)

activity are determined by the ability of the test plasma to induce agglutination of a standardized platelet suspension in the presence of ristocetin. There is a good degree of correlation between the activity of vWF in vitro and its activity in vivo, as assayed by means of the bleeding time.²⁸

von Willebrand Collagen Binding Activity

The vWF-binding activity assay (vWF: Co) assesses the ability of vWF to bind to collagen. The collagen binding assay uses the ELISA sandwich technique to determine vWF activity. The proportion of von Willebrand binding activity should be interpreted in relation to vWF:Ag to distinguish between type 1 and type 2 vWD. A collagen binding defect can be ruled out in the event the proportion of vWF: CB and vWF:Ag is less than one.³⁰ Plasma is added to microtiter wells coated with collagen. The high molecular weight vWF multimers present in the plasma will bind to the collagen according to

TABLE 33-3 Types of von Willebrand's Disease

	Etiology	VWF Antigen	Ristocetin Cofactor	Collagen Binding
Type 1	Decreased production	Decreased	Decreased	Decreased
Type 2A	Decreased HMW multimers, decreased platelet-dependent function	Slightly decreased or normal	Very decreased	Very decreased
Type 2B	Increased affinity for platelet GPIb/IX/V	Slightly decreased or normal	Decreased	Decreased
Type 3	Severe quantitative deficiency	Essentially absent	Essentially absent	Essentially absent
Type 2M	Decreased platelet-dependent function; not related to HMW multimer deficiency	Decreased	Very decreased relative to antigen level	Decreased
Type 2N	Factor VIII binding dysfunction	Normal	Normal	Normal
PseudovWD	Defect of platelet GPIb/IX/V binding site	Normal	Normal	Normal

METHOD 33-14 von Willebrand Factor Antigen

Principle	The ELISA method utilizes a microtiter plate coated with specific rabbit antihuman vWF antibodies to capture the vWF to be measured. Rabbit anti-vWF antibody coupled with peroxidase binds to the remaining free antigenic determinants of vWF, forming the "sandwich." The bound enzyme peroxidase is then detected by its activity on the substrate orthophenylenediamine (OPD) in the presence of hydrogen peroxide or TMB (Fig. 33-10). The reaction is stopped with a strong acid. The intensity of the color produced is directly related to the vWF concentration present in the plasma sample and is read from a standard curve.
Specimen	Sodium citrate anticoagulated plasma
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	The vWF antigen level is decreased in type 1 and type 3 von Willebrand's disease. Type 2 von Willebrand's disease typically shows a normal or slightly decreased antigen with a decreased vWF activity due to a dysfunctional protein, or loss of the functional high molecular weight multimers due to increased proteolysis.
Limitations	The vWF:Ag is an acute-phase reactant, and levels increase above normal during pregnancy, with use of birth control pills, physical exercise, and stress. They also rise with age. Elevated vWF:Ag levels may also be observed when there is injury to the vascular endothelium, such as cancer, fever, in hepatic or renal disorders, during the postoperative period, or with thrombosis and myocardial infarction. It is also worth noting that blood group type-O normally express 25% lower levels of vWF compared with type-A patients. ²⁷ Protein degradation could lead to falsely decreased values.
Reporting Results	Reported in relative percent in relation to normal plasma
Reference Ranges	50% to 150%
Notes	ELISA kits are readily available, and procedures tend to be very similar, only differing in manufacturer-specified reagents.

METHOD 33-15 von Willebrand Activity (vWF:RCO, Ristocetin Cofactor)

Principle	The vWF:RCO assay assesses the interaction between vWF and platelet glycoprotein Ib/IX/V (GPIb/IX/V) where factor VIII/vWF complex promotes agglutination of platelets in the presence of ristocetin. Normal reconstituted lyophilized platelets are mixed with dilutions of control or test plasma. Ristocetin is added, and the rate of aggregation is quantitated. The rate of aggregation is proportional to vWF factor activity. The activity of unknown test samples is extrapolated from a reference graph obtained by testing dilutions of normal pooled plasma.
Specimen	Sodium citrate anticoagulated plasma
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	Patients with von Willebrand's disease range from 0% to 50% activity. The results typically correlate well with vWF:Ag. Normal or increased levels of vWF are found in patients with hemophilia A and Bernard-Soulier syndrome. Certain disease states such as diabetes mellitus, hyperthyroidism, liver disease, chronic renal failure, pregnancy, endothelial cell damage, and disorders of the myeloproliferative syndrome may cause an increase in the level of vWF activity.
Limitations	vWF activity may become normal in individuals with von Willebrand's disease during inflammation or pregnancy, or following transfusion with components rich in factor VIII, despite the prolonged bleeding times. ²⁸ Patients who present with a variant form of von Willebrand's disease may show prolonged bleeding times in the face of decreased to normal vWF levels but increased activity of ristocetin. ²⁹
Formulas	A linear regression equation is determined with the slope of diluted reactions on the y-axis and the % vWF on the x-axis on a semilog plot.
Reporting Results	Reported as a percent of normal activity
Reference Ranges	50% to 150% activity (compared with normal pooled plasma)
Notes	Because variations exist with other clinical cases, two or three separate assays may be necessary before making a diagnosis.

their ability. After washing to remove unbound proteins, anti-human vWF conjugated with horseradish peroxidase is added and will complex with the bound vWF. An HRP substrate is added, and the color produced by cleavage of the substrate is measured by the change in OD at 450 nm.

von Willebrand Factor Multimer Analysis

Once a patient is diagnosed with von Willebrand's disease, determination of the type of deficiency is important to determine proper therapy. Multimer analysis is an electrophoretic technique used to identify the vWD phenotype and

is considered the gold standard of testing in acquired von Willebrand's disease.³¹

Plasma samples are treated with a detergent-buffer and electrophoresed through a low concentration agarose or polyacrylamide gel. VWF multimers separate according to size, with the highest MW multimers closest to the application point. The multimers are transferred to nitrocellulose paper via a Western blot technique, and the blot incubated with a labeled antibody to vWF. The presence of vWF multimers is revealed by addition of a colorimetric substrate, or if a radio-labeled anti-vWF is used, by development of a radiograph. As seen in Figure 33-11, a normal pattern shows bands throughout the gel; type 1 vWD shows a normal pattern of bands with a decreased quantity, and types 2B and 2A show a loss of high and both high and mid molecular weight bands, respectively. A type 3 pattern shows the presence of no vWF. Standardized diagnostic guidelines to accurately interpret and appropriately classify different forms of vWD are necessitated due to the misclassification events.

Molecular Analysis in vWD

Molecular diagnosis of vWD has many confounders in genetic analysis. Molecular defects have been observed in numerous functional domains in the multimeric glycoprotein necessitating multiple tests for diagnosis. The relatively large *vWF* gene spans 178 kb and contains 52 exons. It is highly polymorphic containing numerous SNPs, but these variations in sequence are not associated with bleeding abnormalities. Sequencing is

further complicated with the presence of a homologous partial pseudogene on chromosome 22. Although genetic testing is not preferable in most cases, it is most useful when specific laboratory tests are inconclusive, and an effective treatment remains elusive.³²

Tests to Assess Hereditary Thrombotic Risk

Several hereditary defects have been linked to an increased risk of thrombosis, particularly in the venous system in the form of **deep vein thrombosis (DVT)** and **pulmonary emboli (PE)**. These conditions occur frequently in our population with severe consequences. DVT has an incidence of 2 million per year, which is greater than that of heart attack and stroke combined. PE is the cause of 200,000 deaths per year in the United States, half of which are considered preventable if diagnosed and treated properly and causes 10% of all hospital deaths. By knowing a patient's risk factors for venous thrombosis, anticoagulation can be started when thrombotic risk is increased such as postsurgery and during pregnancy to prevent these fatalities.

Important considerations include:

1. Timing of specimen collection is important when testing for hereditary thrombotic risk factors to avoid mislabeling a patient with a hereditary deficiency. Ideally, a patient should be tested when they are not on any anticoagulants and are nonsymptomatic. Protein C and protein S are vitamin K dependent and will be decreased while a patient is on warfarin. They are also decreased following an acute thrombotic event, and protein S is decreased during pregnancy. Antithrombin is decreased in vivo during heparin therapy. DNA assays for genetic mutations are not affected by disease, and treatments may be performed at any time.
2. Age of the patient when presenting with their first venous thrombotic event should direct the laboratory evaluation of hereditary risk factors. Antithrombin-deficient patients typically have their first event at a relatively young age, in their teenage years or 20s. Protein C- and protein S-deficient patients usually present before age 60. APC-resistant patients may develop their first thrombosis at any age, even after age 60. Risk of thrombosis increases gradually with age in all people, and by age 75 the incidence of venous thromboembolism is estimated at 1 in 100.

Method 33-16: Activated Protein C Resistance/ Factor V Leiden

Activated protein C (APC) resistance may be either hereditary or acquired. The most common cause of hereditary APC resistance is the presence of **factor V Leiden mutation (A506G)**. This mutation is found heterozygous in approximately 5% and homozygous in about 0.02% of the Caucasian population.³³ This inherited defect makes the factor V molecule resistant to the proteolytic activity of activated protein C, thereby negating its regulatory effect on thrombin generation and increasing the potential for the development of thromboses. Owing to possible interferences in the clot-based



FIGURE 33-11 vWF multimer gel. Lane 11. Type 2A showing loss of high and moderate MW multimers. Lane 10. Type 1 showing a normal distribution with decreased quantity. Lane 9. Type 2B showing a loss of high MW multimers. Lane 8. Normal pattern.

METHOD 33-16 Activated Protein C Resistance/Factor V Leiden

Principle	The APC resistance assay is a clot-based assay used most frequently to screen for the presence of factor V Leiden. In this assay, patient plasma is diluted 1:5 or 1:10 in factor V-deficient plasma. This normalizes all other coagulation proteins and makes the reaction dependent on the factor V in the patient sample. Activated protein C is added, and an aPTT or dilute Russell viper venom time (dRVVT) performed. The activated protein C will inhibit factor V procoagulant activity in a normal sample, prolonging the aPTT or dRVVT. A blank reaction is performed at the same time substituting buffer for the APC in the assay. The clotting time including APC is divided by the clotting time of the buffer reaction. A ratio of greater than 2.4 is generally considered normal with a PTT-based assay, although the normal cut-off must be determined with each reagent/instrument system in each laboratory. To test for acquired APC resistance, the assay is performed without dilution in factor V-deficient plasma.
Specimen	Sodium citrate anticoagulated plasma
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	Abnormal resistance to activated protein C is consistent with a ratio of less than 2.0.
Limitations	Patients should not be receiving argatroban or like products. Elevated levels of factor VIII (acute phase reactant) can normalize the aPTT and reduce the effect of activated protein C. Abnormal results may be seen in pregnancy due to decreased levels of protein S and increased levels of factors V and VIII.
Formulas	APC resistance ratio = aPTT with APC:aPTT with no APC
Reporting Results	Reported as a ratio expressed as a number to the tenth place.
Reference Ranges	A ratio <1.5 suggests factor V Leiden is present A ratio of 1.5 to 2.0 is borderline A ratio >2.0 means factor V mutation is unlikely
Notes	DNA-based testing is suggested if the APC resistance test is abnormal to confirm APC resistance.

APC screening assay such as lupus anticoagulants, abnormal results must be confirmed with DNA analysis for the factor V Leiden mutation.

ADVANCED CONTENT

DNA analysis may be performed with polymerase chain reaction (PCR) methods, either as site-specific allele amplification with visualization on an agarose or acrylamide gel for normal and mutant bands or as a restriction fragment length polymorphism (RFLP). It may also be performed via the fluorescence energy transfer method (FRET), an automated system, using the automated Invader[®] technology (Third Wave Technologies, Madison, WI).

Antithrombin Assays

Antithrombin (AT-III) is a naturally occurring inhibitor of blood coagulation and plays an important role in hemostasis. It is an α_2 -globulin that is synthesized in the liver and circulates in the plasma. It is the major plasma inhibitor responsible for neutralizing the activity of thrombin; factors IXa, Xa, XIa, and XIIa; and plasmin. Antithrombin slowly, progressively, and irreversibly inhibits the action of thrombin by forming a 1:1 stoichiometric complex with thrombin. This complex forms when the active serine site of thrombin binds with the arginine site of antithrombin.

The inhibition of thrombin by antithrombin is greatly accelerated by heparin.

Antithrombin activity and antigen can be measured by a variety of techniques. The most frequently used assays are (1) chromogenic substrate assays, (2) micro latex particle immunological assay (LIA), and (3) nephelometry.

Method 33-17: Antithrombin Functional Assay (Activity)—Chromogenic Substrate Assay

Chromogenic antithrombin assays measure the functional levels of antithrombin in plasma via an amidolytic method using a synthetic substrate. These assays may be based on inhibition of thrombin or FXa.

Method 33-18: Antithrombin Immunological Assay (Antigen)—Microlatex Particle Immunological Assay

Immunological antithrombin assays quantitate the amount of antithrombin in plasma.

Protein C Assays

Protein C is a vitamin K-dependent serine protease that functions as a major regulatory protein in the control of coagulation. Thrombin (FIIa) and thrombomodulin convert protein C into its active form of activated protein C. Activated protein C, with its cofactor protein S, is a potent anticoagulant that acts by proteolytically inactivating factors Va and VIIIa, and also enhances fibrinolytic activity in plasma.³⁴ Factors Va and VIIIa are important in accelerating the activation of prothrombin and factor X.

METHOD 33-17 Antithrombin Functional Assay (Activity)

Principle	Plasma containing antithrombin is diluted in the presence of heparin and incubated with excess FIIa (thrombin) or FXa, forming an antithrombin-factor (IIa or Xa)-heparin complex. Heparin bound to antithrombin causes a conformational change in the antithrombin structure. This structural change increases the inhibitory activity of the antithrombin. Any remaining factor (IIa or Xa) catalyzes the cleavage of the chromogenic substrate, resulting in a color change. The release of p-nitroaniline (pNA) is measured by either an endpoint or kinetic method at 405 nm. The absorbance obtained is inversely proportional to the AT activity concentration in the sample and may be quantitated by interpolation from a calibration curve.
Specimen	Sodium citrate anticoagulated plasma
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	<p>Since 1965, antithrombin has been considered important as a result of the description of the first known hereditary deficiency and its consequences; that is, the congenital decrease of the antithrombin level is accompanied by a high frequency of spontaneous thromboembolic disorders.</p> <p>Hereditary qualitative deficiencies are less frequent and are identified by antigenic antithrombin levels being normal while antithrombin activity levels are decreased. In addition to these deficiencies, a number of acquired deficiencies have been described, including DIC, nephrotic syndrome, and liver diseases, and deficiencies resulting from oral contraceptive use, postsurgical state, and after prolonged heparin therapy.</p>
Limitations	<p>If specimen contains heparin (drawn after heparin flush or after receiving heparin), results may be erroneous. Hirudin or argatroban anticoagulants may interfere. Some assays may fail to detect clinically significant variants.</p> <p>This methodology does not detect functional deficiencies by itself. If initial antithrombin result is low, should do confirmatory test on repeat specimen followed by sequencing of the antithrombin (<i>SERPINC1</i>) gene.</p>
Reporting Results	Reported as a percent of normal
Reference Ranges	<p>Neonates 30% to 50%</p> <p>Infants and children 60% to 90%</p> <p>Adults 90% to 120%</p>
Notes	It is useful to differentiate between type I and type II mutations and whether the heparin binding site is affected. The antithrombin gene (<i>SERPINC1</i>) should be sequenced.

METHOD 33-18 Antithrombin Immunological Assay

Principle	A beam of monochromatic light can traverse a suspension of microlatex particles to which specific antibodies have been attached by covalent bonding. If the light is of a wavelength that is much greater than the diameter of the latex particles, it can pass through the latex suspension unabsorbed. However, in the presence of the antigen being tested, the antibody-coated particles agglutinate to form aggregates of diameters greater than the wavelength of the light and the latter is absorbed.
Specimen	Sodium citrate anticoagulated plasma
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	<p>Since 1965, antithrombin has been considered important as a result of the description of the first known hereditary deficiency and its consequences; that is, the congenital decrease of the antithrombin level is accompanied by a high frequency of spontaneous thromboembolic disorders.</p> <p>Quantitative antithrombin deficiencies are the most frequent. They are identified by both the antithrombin antigenic and activity levels being depressed. In addition to these deficiencies, a number of acquired deficiencies have been described, including DIC, nephrotic syndrome, and liver diseases, and deficiencies resulting from oral contraceptive use, postsurgical state, and after prolonged heparin therapy.</p>
Limitations	<p>Some assays may fail to detect clinically significant variants.</p> <p>This methodology does not detect functional deficiencies by itself.</p>
Formulas	There is a direct relationship between the observed absorbance value at 570 nm and the concentration of the antigen being measured. Antithrombin concentration can be directly derived from a correlating standard curve of varying known concentrations of antithrombin reagents.
Reporting Results	Reported as a percent of normal

METHOD 33-18 Antithrombin Immunological Assay—cont'd

Reference Ranges	Neonates 30% to 50% Infants and children 60% to 90% Adults 90% to 120%
Notes	If initial antithrombin result is low, should do confirmatory test on repeat specimen followed by sequencing of the antithrombin (<i>SERPINC1</i>) gene.

ADVANCED CONTENT

Components of the protein C system include proteins C and S, C4b-binding protein, thrombomodulin, activated protein C inhibitor (plasminogen activator inhibitor-3), and thrombin. Protein S, also a vitamin K-dependent factor, is a necessary cofactor in the reaction in which factor Va is inactivated by protein C. Laboratory diagnosis of a protein C deficiency is performed using clot-based assays, ELISA (immunological), and chromogenic assays.³⁴ Sequence analysis of the *PROC* gene is also available. Erroneous results are possible using clot-based assays due to the effects of circulating inhibitors, high levels of FVIII, and by FV-Leiden mutations. The aPTT and the PT are not sensitive to decreases in protein C levels.

Method 33-19: Protein C Immunological Assay (Antigen)

This assay is not generally used to identify a deficiency; rather, this approach detects low levels of activity and the dysfunctional form of the protein.

Method 33-20. Protein C Functional Assays (Activity)—Chromogenic Substrate Assay

This assay measures the response of protein C to Copperhead snake venom.

Method 33-21: Protein C Clot-Based Assay

The clot-based assay determines the ability of APC to prolong the aPTT test.

Protein S Assays

Protein S (PS) is a vitamin K-dependent cofactor for the anti-coagulant and proteolytic effects of activated protein C. In normal plasma, about 60% of protein S is complexed with the C4bBP, whereas the other 40% is in free form. PS bound to C4b-binding protein (C4bBP) has little or no cofactor activity and only free protein S acts as a functional cofactor.³⁷ There are three "activity" assay approaches to test for protein S deficiency. First, clotting assays evaluate the function of PS as a cofactor. These assays are affected by numerous confounders thus should not be the primary evaluation method. There is also the Protein S Free Antigen Assay that measures the unbound or functional protein S. This assay has shown to be the most reliable indicator in protein S deficiency. Lastly, the Protein S Total Antigen assay measures both free and bound protein S. This assay can differentiate different types

METHOD 33-19 Protein C Immunological Assay (Antigen)

Principle	A microtiter plate coated with specific rabbit antihuman protein C antibodies captures the protein C antigen to be measured. Rabbit antiprotein C antibody coupled with peroxidase then binds to the remaining free antigenic determinants of protein C, forming a "sandwich." The bound enzyme peroxidase is then detected by its activity on the substrate ortho-phenylenediamine in the presence of hydrogen peroxide or TMB. The reaction is stopped with a strong acid.
Specimen	Sodium citrate anticoagulated plasma
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	Protein C deficiency has been described as a risk factor in thromboembolic disease. Immunological assays may have undetectable levels of protein C with congenital homozygous deficiency, and falsely elevated levels of protein C may be due to numerous clinical conditions. ³⁵
Limitations	Immunological analysis is very sensitive but does not detect functional defects. Some inhibitors can be detected with this methodology.
Formulas	The intensity of the color produced is directly proportional to the protein C antigen concentration present in the plasma sample. Results are derived from a standard curve.
Reporting Results	Reported as IU/dL
Reference Ranges	Infants to teens 40 to 60 IU/dL Adults 65 to 135 IU/dL
Notes	Mutational analysis of the protein C (<i>PROC</i>) gene should be performed when protein C deficiency is suspected.

METHOD 33-20 Protein C Chromogenic Substrate Assay (Activity)

Principle	Protein C in plasma is activated by a specific enzyme from Southern Copperhead snake venom. The amount of activated protein C is determined by the rate of hydrolysis of the synthetic substrate S-2366. The amount of pNA (yellow color) release measured at 405 nm is proportional to the protein C level.
Specimen	Sodium citrate anticoagulated plasma
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	Protein C deficiency is an autosomal dominant disorder and is associated with an increased risk of VTE.
Limitations	Heparin levels up to 3.0 IU/mL do not interfere with the assay. Elevated levels of hemoglobin, bilirubin, or lipid may interfere with the assay, and results from severely hemolyzed, icteric, or lipemic specimens should be interpreted accordingly. The chromogenic activity assay may overestimate the protein C activity in patients on oral anticoagulant therapy due to the activity of noncarboxylated forms of the protein. Chromogenic assays have a high sensitivity, detect most functional defects, and are not affected by clot-based interferences like lupus anticoagulant, factor VIII levels, or the presence of the factor V Leiden mutation but are unable to detect rare mutations.
Formulas	The quantity of protein C is determined based on the rate of the color change in the test sample due to the chromogenic substrate. Protein C concentrations are derived from a standard curve.
Reporting Results	Reported as IU/dL
Reference Ranges	Infants to teens 40 to 60 IU/dL Adults 65 to 135 IU/dL
Notes	The chromogenic assay is not dependent on protein S levels. Mutational analysis of the protein C (<i>PROC</i>) gene should be performed when protein C deficiency is suspected.

METHOD 33-21 Protein C Clot-Based Assay

Principle	Protein C is activated in the presence of a specific activator, such as snake venom. Protac is one such toxin, extracted from the venom of copperheads, that remains the gold standard in protein C detection and quantification. ³⁶ The resulting activated protein C inactivates factors Va and VIIIa, and thus prolongs the aPTT compared with the baseline value.
Specimen	Sodium citrate anticoagulated plasma
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	Low levels of protein C are observed at birth as a result of liver immaturity. In adults, the protein C level appears to be independent of age and sex. Hereditary protein C deficiency can be classified as type I or type II, according to the levels of protein C activity and antigen measured. Acquired deficiencies of protein C are observed in hepatic disorders such as hepatitis and cirrhosis, vitamin K deficiency, DIC, and oral anticoagulant therapy. In these cases, the interpretation of test results is difficult if the patient has had a history of thromboses and is receiving anticoagulant treatments.
Limitations	Heparin does not affect test results when present at a concentration less than 1.0 IU/mL. Higher levels of heparin may lead to an overestimation of the protein C level by causing an additional prolongation of the aPTT. The presence of lupus anticoagulants may also prolong the clotting time, causing a falsely elevated result. Misleading decrease may be seen with factor V Leiden factor mutation and in cases of activated protein C resistance.
Formulas	Protein C activity is determined based on the degree of prolongation of the aPTT compared with a calibration curve prepared from dilutions of normal pooled plasma.
Reporting Results	Reported as IU/dL
Reference Ranges	Infants to teens 40 to 60 IU/dL Adults 65 to 135 IU/dL
Notes	Mutational analysis of the protein C (<i>PROC</i>) gene should be performed when protein C deficiency is suspected.

of PS deficiency but should not be used as a primary test methodology due to conditions that express normal levels of protein S.³⁸ Total and free protein S can also be measured via latex immunoassay (LIA) or ELISA methodology. The immunological approach enables testing for specific PS mutations.

Method 33-22 Protein S Functional Assay (Activity)—Clotting Assay

The functional protein S assay is based on the observation that activated protein C will inactivate factors Va and VIIIa in the presence of protein S.

METHOD 33-22 Protein S Functional Clot-Based Assay (Activity)

Principle	Diluted plasma is mixed with protein S-deficient plasma, which is then activated in a one-stage assay that uses factor Xa, phospholipid, and activated protein C. The activator may be aPTT-based, FX-based, or PT-based depending on the kit used. A linear relationship exists between the concentration of protein S and the prolonged clotting time. Normal plasma added to protein S-deficient plasma causes a prolonged clotting time proportional to the amount of normal plasma added.
Specimen	Sodium citrate anticoagulated plasma
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	Protein S deficiency is an autosomal dominant disorder with increased risk of venous thromboembolism (VTE). Homozygous protein S deficiency may be present in neonates exhibiting extensive cutaneous hemorrhaging. Type II deficiency is detected with a functional assay.
Limitations	Negative results are not always conclusive and further testing may be required. The presence of heparin and high levels of factor VIII may interfere with this assay, as do lupus anticoagulants. Clot-based tests for protein S deficiency should be used as a screening test. Results obtained from this assay are not specific and erroneous results may be obtained when a factor V-Leiden factor or lupus anticoagulant is present. ³⁹ Protein S may appear decreased due to high levels of C4b, oral contraceptives, and in samples more than 8 hours old. ⁴⁰
Formulas	Functional protein S determinations are obtained by comparing the clotting time of normal plasma with that of the patient plasma on a reference curve.
Reporting Results	Reported as U/dL
Reference Ranges	Neonate levels low due to vitamin K—dependent clotting factors Males >70 to 140 U/dL Females >60 to 130 U/dL
Notes	Genetic sequencing of the <i>PROS1</i> gene is available and results compared with documented mutations on the International Society of Thrombosis and Haemostasis (ISTH) registry.

Protein S Immunological Assay (Antigen)

Previously described latex immunoassays (LIA) for free and total protein S antigen determinations are commercially available and may be performed on automated analyzers.

Commercial assays are also available that use the ELISA sandwich technique with monoclonal capture antibodies to determine free and total protein S levels.

Prothrombin G20210A (Factor II) Mutation

A G-to-A substitution in nucleotide position 20210 of the 3' untranslated region of the prothrombin gene is responsible for a common polymorphism of factor II protein. Individuals who inherit one abnormal allele are considered heterozygous and those with two mutant alleles are homozygous for G20210A. The presence of one prothrombin G20210A allele (heterozygous) is associated with at least a three- to six-fold increased risk of deep venous thrombosis for all ages and both genders. Homozygous deficiency further increases risk. Risk of thrombosis is further increased by up to 80 times normal in individuals who carry both the prothrombin defect and factor V Leiden. Increased levels of prothrombin may be seen in individuals that carry the prothrombin G20210A mutation and appear to be the cause of increased risk of thrombosis, though the increased level may be in the upper limit of the normal range when measured with the one-stage clotting activity assay for factor II. Prothrombin antigen and/or activity levels cannot be used to test for the presence of the mutation, however,

as these assays cannot distinguish high normal factor II levels from those caused by the G20210A polymorphism.

As with factor V Leiden, DNA testing for the G20210A polymorphism may be carried out with PCR methods using either site-specific allele amplification or RFLP. The mutation may also be tested with FRET technology on the Invader[®] system.

Tests for the Evaluation of Lupus Anticoagulants

Lupus anticoagulants are acquired antibodies that are directed at phospholipid-protein complexes. Lupus anticoagulants differ from naturally occurring inhibitors such as antithrombin, α_2 -macroglobulin, α_1 -antitrypsin, and C1 esterase and must be differentiated from anticoagulants such as heparin and coumarin analogues. Most lupus anticoagulants are inhibitors or autoantibodies of the IgG class whose inhibitory effects demonstrate specific activity against phospholipid-protein complexes (such as B2GPI or the prothrombin complex).

Some lupus anticoagulants that have been detected thus far have been encountered in patients with the following conditions: hemophilia A (factor VIII deficiency), Christmas disease (factor IX deficiency), DIC, pregnancy, systemic lupus erythematosus (SLE), plasma cell dyscrasias, Waldenström's macroglobulinemia, and advanced age.

Since lupus anticoagulants (LAs) are antibodies (IgG, IgM, IgA, or a combination) directed against phospholipid-protein

complexes, they may prolong *in vitro* phospholipid-dependent coagulation tests. First recognized in patients with SLE, LAs have been identified in a variety of disorders including malignancies, infections, and autoimmune disorders, as well as after drug therapy. The presence of an LA is usually not associated with a bleeding problem unless accompanied by thrombocytopenia, factor II deficiency, platelet dysfunction, or drug administration (e.g., aspirin). The LA, however, has been identified as a risk factor for venous and arterial thrombosis and recurrent spontaneous abortions.⁴¹

The laboratory diagnosis of a lupus inhibitor is critical in terms of distinguishing it from other specific factor inhibitors and to identify patients at potential risk for thrombotic problems. The Systemic Lupus International Collaborating Clinics (SLICC) 2012 has defined the following criteria to make a diagnosis of a lupus anticoagulant.⁴¹

Diagnosis of SLE is complex and involves correlation of a total of at least four laboratory and clinical criteria. The laboratory testing is listed as follows:

1. Positive ANA
2. Positive anti-dsDNA (except ELISA) on ≥ 2 occasions
3. Anti-Sm
4. Antiphospholipid antibody (including lupus anticoagulant, false positive RPR, anticardiolipin, antibeta2glycoprotein1)
5. Low complement (C3, C4, or CH50)
6. Direct Coombs' test in the absence of hemolytic anemia
7. LAs must be carefully distinguished from other coagulopathies that may give similar laboratory results or may occur concurrently with LAs. The clinical history may be helpful in differentiating LAs from these other possibilities.

Suspicion of an LA is most often aroused by an unexplained prolongation of the APTT that is not corrected by the addition of an equal volume of normal plasma. It should be noted that the time-dependent inhibition, which is usually considered an indicator of factor VIII inhibitors, has been seen with LAs, as well as factor V inhibitors. Confirmatory tests to identify an LA include those that utilize a low concentration of phospholipid in the test system, thereby increasing the LA effect such as the tissue thromboplastin inhibition test (TTIT), dilute Russell's viper venom time (dRVVT), and the kaolin clotting time (KCT), or those that increase the phospholipid, thereby neutralizing the LA effect, such as the platelet neutralization procedure. Another type of confirmatory test depends on the presence of hexagonal phase phospholipids and can also be used. It is important to note that the confirmatory test selected should correspond to the screening test that is abnormal. For example, if the APTT is abnormal, the platelet neutralization procedure would be a logical confirmatory test.

Confirmatory Tests for Lupus Anticoagulants

There are several confirmatory tests for the LA, including platelet neutralization procedure, hexagonal phospholipid neutralization assay, anti- β 2GPI assay, anticardiolipin assay, KCT, tissue thromboplastin inhibition test, and dRVVT. The first four are described in this section.

Method 33-23: Platelet Neutralization Procedure

This assay is used in conjunction with other coagulation tests in determining the presence of a lupus anticoagulant.

METHOD 33-23 Platelet Neutralization Procedure

Principle	The lupus anticoagulant (LA) causes prolongation of the aPTT phospholipid-dependent coagulation tests (aPTT, DRVVT, etc.). The platelet neutralization procedure (PNP) is based on the ability of fractured platelets to absorb LA <i>in vitro</i> and correct abnormal clotting times. ⁴² The disrupted platelet membranes present in the freeze-thawed platelet suspension neutralize phospholipid antibodies present in the plasma of patients with LA. After the patient plasma is mixed with the freeze-thawed platelet suspension, the aPTT will be shortened compared with the original baseline aPTT.
Specimen	Sodium citrate anticoagulated plasma and washed freeze, thawed platelets
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	A correction of the baseline aPTT of a defined amount of time (i.e., 3 to 5 seconds or more) by the platelet suspension compared with the control is indicative of the presence of an LA.
Limitations	Heparin can cause a false-positive PNP. FV deficiency or inhibitors may produce a false positive PNP interpretation. The presence of other coagulopathies must be eliminated through other coagulation tests. Coagulation assays that have a phospholipid-dependent reaction are affected by the presence of residual platelets in platelet-poor plasma. Because LAs are directed against phospholipids, the relative concentration of any platelet phospholipid contained in the sample affects the sensitivity of the assay for detection of the LA.
Formulas	Determine the difference between the initial aPTT and the aPTT performed when mixed with fractured platelets added.
Reporting Results	Reported in seconds
Reference Ranges	>3 to 5 seconds difference between aPTT analysis means LA present; no change in aPTT results is considered negative for LA
Notes	The PNP should be considered when the aPTT is increased and not corrected in mixing studies.

Hexagonal Phospholipid Neutralization Assay

The hexagonal phospholipid neutralization assay uses the same principle as the PNP assay. Normalization of the aPTT in the presence of added phospholipid, but this assay specifically uses a phospholipid in a hexagonal conformation. Neutralization by this hexagonal form in an assay with a very lupus-anticoagulant sensitive aPTT reagent is a more sensitive confirmation test than the PNP.

Specimen collection, centrifugation, and processing are critical when testing for the presence of an LA. LAs demonstrate considerable heterogeneity and show variable differences in sensitivity and responsiveness of the reagent. This makes the selection and interpretation of an appropriate confirmatory test challenging. Despite differences in testing methodology and reagents currently available, most of these anticoagulants can be detected and identified in the routine laboratory setting.

Anti-Phospholipid Antibody Assays

Antibeta-2 glycoprotein 1 (anti- β 2 GPI) and anticardiolipin antibodies (ACAs) are antiphospholipid immunoglobulins that are IgG, IgM, IgA, or a combination. Antiphospholipid antibodies are a heterogeneous group of autoantibodies including ACA, LA, beta-2 glycoprotein-1 (β 2GP-1), antiprothrombin, and antiphosphatidylserine (APTS). Some patients with elevated anti- β 2GPI and/or ACAs have been reported to also have an LA. Several studies have shown that patients with the LA and the closely related anti- β 2GPI/ACA are prone to recurrent venous and arterial thrombosis, recurrent spontaneous abortions, and thrombocytopenia.⁴¹ This tendency has been described as the antiphospholipid syndrome (APS). The criteria for diagnosing this syndrome are divided into clinical and laboratory groups. APS is present if at least *one* of the clinical criteria and *one* of the laboratory criteria that follow are met:

1. Clinical criteria: vascular thrombosis or pregnancy morbidity
2. Laboratory criteria:
 - a. Lupus anticoagulant present in plasma on two or more occasions at least 12 weeks apart.
 - b. Anticardiolipin antibody-, IgG-, or IgM-positive in medium or high titer on two or more occasions at least 12 weeks apart
 - c. Anti- β 2GP-1 antibody, IgG, or IgM present on two or more occasions at least 12 weeks apart.

The positive laboratory criteria and the clinical criteria should occur within 12 weeks to 5 years of each other.⁴¹ ELISA techniques are available for measuring Antiphospholipid antibodies such as ACA, anti- β 2GP-1, antiphosphatidylserine, and antiprothrombin antibodies. The commercial kits use either cardiolipin or a mixture of negatively charged phospholipids as capture antigens depending on the desired specificity of the kit.

Tests for Fibrinolysis

D-Dimer Quantitative Test

Under the action of thrombin, fibrinogen is cleaved to give rise to fibrin monomers. These monomers form polymers, which are stabilized by factor XIII, forming covalent

cross-linkages in the D domain to produce an insoluble fibrin clot. Plasmin, a potent clot-lysing enzyme, attacks fibrin clots as well as fibrinogen in the body. Unlike the action of plasmin on fibrinogen, which produces FDPs, its action on the fibrin clot leads to the generation of cross-linked fibrin containing **D-dimer**. D-dimer assays may be performed using an ELISA method or a latex-agglutination immunoassay technique that is automated. The latex particles provided in the D-dimer LIA test are coated with antihuman D-dimer monoclonal antibodies. When plasma containing D-dimer is added to the latex reagent, the latex particles agglutinate, resulting in a change in OD that is monitored at 570 nm. There are two commonly used reporting units for D-dimer: fibrinogen equivalent units (FEU) or D-dimer units, where 1 D-dimer unit equals 2 FEU. Normal D-dimer levels in plasma are less than 250 ng/mL D-dimer units (0.5 mcg/mL FEU).

One of the most important aspects of this assay is its negative predictive value when ruling out venous thromboembolism. When used in an emergency department and when the patient evaluation includes a pretest probability screening by the clinician, the D-dimer assay with an appropriate cut-off value of <500 mcg FEU/L had a negative predictive value for a venous thromboembolism at 96% over imaging (lung scan and duplex ultrasonography).⁴² An elevated D-dimer level may be seen in clinical situations where active thrombosis is occurring, such as DIC, deep vein thrombosis, and pulmonary embolism.

The advantage of the D-dimer assay over the FDP assay is that there is no interference from fibrinogen, so the test can be run on citrated plasma without necessitating collection in a special tube. It has limited usefulness, however, in the evaluation of primary fibrinolysis because it will detect only breakdown products from cross-linked fibrin and not those from fibrinogen. Other laboratory tests that may be useful in evaluation of the fibrinolytic system include fibrin monomers, ethanol gelation test, protamine sulphate test, and euglobulin lysis time.

Method 33-24: Euglobulin Lysis Time

The euglobulin lysis time (ELT) test is a global screening assay that evaluates the fibrinolytic system function by measuring the time it takes for an *in vitro* clot to dissolve in the absence of plasmin inhibitors. There is no agreement concerning the "gold standard" for fibrinolytic assessment.⁴⁴

Method 33-25: Fibrin Degradation Products: Latex Agglutination Method

Plasmin proteolytically cleaves fibrin(ogen) into fragments X and Y, known as early degradation products, and fragments D and E, known as late degradation products. These FDPs share antigenic determinants with both fibrin and fibrinogen, thus allowing for detection by immunological methods using antisera to highly purified preparations of human fibrinogen fragments D and E. Measurement of FDPs provides an indirect assessment of fibrinolysis.

Generally, elevated levels of FDPs are associated with thrombotic episodes such as myocardial infarction, pulmonary emboli, and deep vein thrombosis, as well as with certain complications of pregnancy.

METHOD 33-24 Euglobulin Lysis Time

Principle	<p>A sample of citrated plasma is processed to separate the euglobulin fraction of proteins, with acid precipitation and then dissolution in saline. The precipitate contains fibrinogen, plasminogen, and plasminogen activators but no plasmin inhibitors such as alpha-2-antiplasmin and alpha-2-macroglobulin. Calcium chloride is added to the redissolved precipitate to form a clot. The time required for the intrinsic plasmin to lyse the fibrin clot equates to the euglobulin lysis time.</p> <p>The lysis time is defined as the time from the clot dissolution curve, corrected for the patient blank OD.</p>
Specimen	Sodium citrate anticoagulated plasma
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	<p>A shortened ELT indicates increased fibrinolytic potential in the patient, and conversely a prolonged ELT is indicative of decreased fibrinolytic potential.</p> <p><60 minutes represents hyperfibrinolysis.</p> <p>>240 minutes indicates hypofibrinolysis.</p>
Limitations	<p>The diagnostic potential of euglobulin lysis time is limited by the extreme variation in lysis times among healthy individuals. Both hypofibrinogenemia and factor XIII deficiency may result in a shortened lysis time. In the case of hypofibrinogenemia, the shortened time is due to the decreased amount of fibrin to be lysed. In factor XIII deficiency, the clot is not stabilized by covalent cross-linking of fibers and can be readily lysed by plasmin.</p> <p>Traumatic venipuncture, prolonged stasis, incorrect sample preparation, and elevated fibrinogen levels may invalidate test results.</p>
Reporting Results	Reported in minutes
Reference Ranges	90 to 240 minutes
Notes	<p>This assay may be read manually or via microtiter wells that are monitored at an OD of 405 nm as the clot lyses.⁴⁵</p> <p>A Thromboelastogram often serves to monitor fibrinolysis and is a considerably more rapid test to perform than the Euglobulin Lysis Time.</p>

METHOD 33-25 Fibrin Degradation Products: Latex Agglutination Method

Principle	<p>The latex agglutination method is a direct agglutination slide test for the detection and semiquantitation of FDPs. Latex particles in glycine buffer are coated with specific antibodies to human fibrinogen fragments D and E. The presence of FDPs in either serum or urine will cause the latex particles to clump, yielding macroscopic agglutination. An approximate concentration of FDPs in the sample can be determined by testing the sample at different dilutions. Thrombin is initially added to the patient sample to ensure complete clotting and total removal of fibrinogen. The addition of proteolytic inhibitor, soybean trypsin, prevents <i>in vitro</i> activation of the fibrinolytic system.</p>
Specimen	Sodium citrate anticoagulated plasma
Procedure	See the required equipment and steps for this procedure at www.fadavis.com
Interpretation	<p>The test is sensitive to values of 2 mcg of FDPs per milliliter. The presence of agglutination in position 1 indicates the presence of FDPs in a final concentration greater than 10 mcg/mL. The presence of agglutination in position 2 indicates the presence of FDPs in a final concentration of greater than 40 mcg/mL. For the test to be valid, agglutination is present in position 2, it must also be present in position 1 on the slide. Agglutination in tube 1 and lack of agglutination in tube 2 indicate FDPs greater than 10 mcg/mL but less than 40 mcg/mL.</p>
Limitations	<p>The latex agglutination assay has been documented to give false-positive results with sera from patients with rheumatoid arthritis. Trace amounts of FDPs occur in the blood of normal healthy adults and children as a result of physiological fibrinolysis.</p>
Reporting Results	Reported as semiquantitative mcg/mL
Reference Ranges	The mean normal level of serum is 4.9 ± 2.8 mcg FDPs per milliliter.
Notes	The normal value may be elevated during exercise and stress.

The assay is of value in the differential diagnosis of patients with specific types of kidney diseases. Quantitation of urine FDP levels provides a useful clinical means of monitoring glomerulonephritis and kidney rejection following transplantation.

The detection of FDPs is of significant clinical value in assessing patients with DIC. A positive test result, accompanied by an elevated PT and aPTT and a decrease in platelet count and possibly fibrinogen concentration, is suggestive of DIC.

Markers of Coagulation Activation and Thrombin Generation

Thrombosis formation can be a life-threatening situation and diagnosis is imperative to reduce patient morbidity and mortality. Currently clinical presentation and a D-dimer test can be used to rule out thrombosis but cannot be used to confirm it. Research strives to identify biomarkers specific to clot formation for definitive diagnostic information. New assays employing molecular markers have been identified and may provide specific and pertinent data for early diagnosis and management of coagulation disorders.⁴⁶

Before thrombin becomes biologically available, there is a generation of biochemical markers in the blood. Identification of biomarkers provides the opportunity for early and specific detection of a thrombotic event in various clinical presentations. These markers of coagulation activation can be employed in three diagnostic applications:

1. Diagnosis of spontaneous thrombosis
2. Prognosis and follow-up of thrombotic disease
3. Monitoring of anticoagulant therapy

Under normal conditions, all individuals produce measurable quantities of molecular markers of coagulation activation; however, significantly increased levels have been observed in persons with deep venous thrombosis, pulmonary emboli, DIC, and other thrombotic abnormalities. Because of their short half-lives, activation peptides demonstrate blood activation only as long as the thrombotic process is ongoing.

Detection and quantification of molecular biomarkers offer several advantages compared with the conventional

coagulation tests. First, molecular markers are specific indicators of the activation of coagulation and fibrinolysis whereas conventional assays measure deficiencies of the clotting mechanism. In addition, markers are ultrasensitive and are able to detect minute changes in the components of hemostasis. Nonetheless it should be noted that limitations to biomarker detection exist in the inability to evaluate all components of the clotting process to fully evaluate the true biological process that is occurring. A multifaceted approach encompassing the clinical presentation, the components involved, and the biomarkers present is advised to accurately diagnose a thrombotic event.

Prothrombin fragment 1 + 2 (PFI + 2), thrombin-antithrombin complex (TAT), and fibrinopeptide A are current markers that can be measured to determine the generation of thrombin. Markers observed in abnormal levels in cardiovascular patients are under investigation for correlation for thrombosis.⁴⁶ ELISA assays are currently available to test for these thrombotic markers.

Global thrombin generation assays that use either a chromogenic or fluorometric method and measure the lag time to thrombin generation, the rate of thrombin formation, and the total amount of thrombin generated are in development and on the horizon for the clinical laboratory.

CRITICAL THINKING QUESTION

33-3 Which coagulation tests should be included when assessing a patient for potential DIC?

SUMMARY CHART

- The bleeding time is a screening procedure used to assess *in vivo* platelet function.
- Platelet aggregation studies measure the platelets' ability to adhere to each other and release endogenous ADP.
- The activated partial thromboplastin time (aPTT) is a screening test used to evaluate the intrinsic pathway of coagulation, which measures all the plasma coagulation factors with the exception of factors VII and XIII.
- The prothrombin time (PT) is a valuable screening procedure used to indicate possible factor deficiencies of the extrinsic pathway.
- The PT test is sensitive to the vitamin K-dependent factors of the extrinsic pathway (factor VII) and common pathway (factors II and X) and is used as a means of monitoring oral anticoagulant therapy.
- The PT is prolonged in individuals with a factor deficiency involving a single factor (i.e., patients with a congenital deficiency) or involving multiple factors (i.e., patients with acquired deficiencies such as liver

disease, oral anticoagulant therapy or patients with vitamin K deficiency).

- The international normalized ratio (INR) is used to standardize prothrombin time results in patients receiving oral anticoagulant therapy and should be reported along with the prothrombin time results.
- The thrombin time (TT) measures the conversion of fibrinogen to fibrin. It is affected by abnormal levels of fibrinogen, dysfibrinogenemia, and the presence of circulating anticoagulants (antithrombins) such as heparin and fibrin(ogen) degradation products (FDPs).
- The reptilase time is similar to the thrombin time in that it measures the conversion of fibrinogen to fibrin. The advantage of reptilase time is that it is not affected by heparin.
- It is the property of the factor VIII-vWF complex that is responsible for agglutination of platelets in the presence of ristocetin.
- The inhibition of thrombin by antithrombin is greatly accelerated by heparin.

(continued)

SUMMARY CHART—cont'd

- Functional antithrombin (activity) is measured by chromogenic assays. Immunological antithrombin (antigen) is measured via Laurell rocket, radial immunodiffusion, or latex immunoassay techniques.
- A number of acquired antithrombin deficiencies have been described, including disseminated intravascular coagulation (DIC), nephrotic syndrome, and liver diseases; deficiencies may also occur with oral contraceptive use, post-surgically, and following prolonged heparin therapy.
- Protein C is a vitamin K–dependent serine protease that functions as a major regulatory protein in the control of coagulation. Activated protein C is a potent anticoagulant that acts by proteolytically inactivating factors Va and VIIIa. Protein C deficiency has been described as a risk factor in thromboembolic disease.
- Activated protein C resistance (APC-R), which is most commonly the result of a mutation in the factor V gene, is known as factor V Leiden. This mutation makes the factor V molecule resistant to the proteolytic activity of activated protein C, thereby negating its regulatory effect on thrombin generation and increasing the potential for the development of thromboses.
- Protein C activity can be measured with chromogenic or clot-based assays. Protein C antigen is usually measured by enzyme-linked immunosorbent assay (ELISA) techniques.
- Protein S is a cofactor in the protein C pathway. It is a vitamin K–dependent factor, and is necessary in the reaction in which factors Va and VIIIa are inactivated by protein C.
- Circulating anticoagulants may be detected by abnormalities in the PT or aPTT, or both, and may be caused by either specific factor inhibitors such as factor VIII antibodies, or nonspecific, such as the lupus anticoagulant.
- Lupus anticoagulants (LAs) are antibodies (IgG, IgM, IgA, or a combination) directed against phospholipids, thereby prolonging in vitro phospholipid-dependent coagulation tests. First recognized in patients with systemic lupus erythematosus (SLE), LAs have been identified in a variety of disorders, including malignancies, infections, and autoimmune disorders, as well as after drug therapy.
- There are several confirmatory tests for LAs, including platelet neutralization procedure, hexagonal phospholipid assay, anti- β 2GPI, anticardiolipin assay, kaolin clotting time, tissue thromboplastin inhibition test, and dilute Russell's viper venom test.
- Fibrinolysis can be evaluated by measuring D-dimer, or FDPs. The quantitative D-dimer is useful in its negative predictive value for ruling out DVT and PE. The FDP assay measures both fibrin and fibrinogen breakdown products, whereas the D-dimer measures only fibrin breakdown products that have been cross-linked in a fibrin clot.
- Prothrombin fragment 1 + 2 (PF1 + 2) and thrombin-antithrombin complex (TAT) are molecular markers that can be measured to determine the generation of thrombin.
- TEG is a point of care platform that can assess clotting time and rate, clot strength, and fibrinolysis.

CASE STUDY 33-1

A 29-year-old woman with a history of three spontaneous abortions is seen in a reproductive clinic where blood is collected, and the following screening results obtained:

PT = 12.0 sec (reference range 10.0–13.0)

aPTT = 45 sec (reference range 26–35)

Thrombin time = 15 sec (reference range 13–18)

Because of the prolonged aPTT, further testing is performed:

1:1 mix with normal pooled plasma = 42 sec

1:1 mix with saline = 53 sec

Normal pooled plasma control = 30 sec

QUESTIONS

1. Based on the patient's PT and aPTT results, what could be a possible hemostasis abnormality?
2. Because the patient's aPTT did not correct during the mixing study, what are possible conclusions?
3. What confirmatory tests could be run to diagnosis this patient?

ANSWERS

1. The patient's aPTT is prolonged, while the PT and fibrinogen activity are normal. An abnormal aPTT can be caused by either a deficiency of one of the intrinsic pathway factors or an inhibitor to the clotting reaction.
2. When the patient's plasma was mixed with an equal volume of normal pooled plasma, the aPTT shortened by only 3 seconds, which is not a correction. The plasma mixed with saline clotted only slightly. The lack of correction leads one to think that an inhibitor may be present, because if this were a factor deficiency, the aPTT would have corrected when mixed with normal pooled plasma. The most common circulating inhibitor are lupus anticoagulants, which align with the patient's history as they have a strong clinical correlation to spontaneous abortion.

CASE STUDY 33-1—cont'd

- To confirm the presence of a nonfactor-specific inhibitor, the dRVVT, hexagonal phospholipid neutralization assay and anti- β 2GPI antibodies were performed:
 DRVVT screen = 50 sec (reference range 25–35)
 DRVVT confirm = 25 sec; DRVVT ratio = 2.0 (reference range <1.2)
 Hexagonal phospholipid neutralization = 30 secs (reference range <8 sec)

Anti- β 2GPI IgG = 45 units (<21), IgM = 34 units (<21), IgA = 24 units (<21)

These results are consistent with the presence of an antiphospholipid antibody. The patient was started on low molecular weight heparin therapy as soon as her next pregnancy was confirmed and resulted in the birth of a full-term infant.

CASE STUDY 33-2

A 35-year-old man presents to the emergency room with swelling and pain in the left leg for the past 3 days. An ultrasound evaluation was done and showed the presence of a deep vein thrombosis in the left leg. The patient was immediately started on unfractionated heparin therapy with the addition of warfarin after 24 hours. Blood was collected 3 days after admission and a thrombophilia evaluation requested. The results were:

aPTT = 65 sec (therapeutic for UFH is 60–90 sec)

PT = 24.0 sec (11–13 sec)

INR = 2.0

AT activity = 58% (70%–130%)

Protein S activity = 45% (60%–140%)

Protein C activity = 52% (60%–140%)

APC resistance ratio = 3.0 (<2.4)

QUESTIONS

- Why might this patient's PT and aPTT be prolonged?
- Explain the decreased AT activity.
- Are these results diagnostic of a hereditary thrombophilic defect?

ANSWERS:

- The time the sample was collected in relationship to clinical symptoms and administration of therapeutic agents is critical to correct interpretation of the results for thrombophilic defects. In this case, both the aPTT and PT are prolonged, but the sample was collected after administration of heparin and warfarin. Protein S

and protein C are decreased likely due to consumption of these proteins during an acute thrombotic event and perhaps secondarily to the early effects of administration of warfarin.

- The antithrombin activity is decreased. This protein is also consumed during an acute thrombotic event and is decreased in vivo with heparin administration.
- Though not always possible, the ideal time to test a patient for risk factors for hereditary thrombophilia is when they are asymptomatic and on no anticoagulant therapy. In addition, testing family members of the patient may aid in determining a hereditary defect when it is not possible to accurately test a symptomatic patient.

FOLLOW-UP:

Seven months after discharge, the patient returned for additional testing. He had been off warfarin for 1 month and symptom-free since discharge. The results of this sample were:

aPTT = 26.0 sec

PT = 12.0 sec

Protein S activity = 85%

Protein C activity = 50%

Protein C antigen = 53% (60%–140%)

APC resistance ratio = 2.9

On return, all test results were within normal limits except for protein C activity. The patient's parents and brother were tested and a hereditary protein C deficiency confirmed.

REVIEW QUESTIONS

- What is measured by a PFA-100?
 - Platelet number
 - Platelet function
 - Intrinsic and extrinsic coagulation systems
 - Fibrinolysis
- Which platelet aggregation result would be characteristic for patients with Bernard-Soulier syndrome?
 - Incomplete aggregation with ADP
 - Primary wave of aggregation in response to collagen
 - Abnormal ristocetin-induced platelet aggregation
 - Primary wave of aggregation with epinephrine

REVIEW QUESTIONS—cont'd

3. Which test measures the extrinsic pathway of coagulation, measures factor deficiencies, and monitors oral anticoagulation therapy?
 - a. Activated partial thromboplastin time
 - b. Prothrombin time
 - c. Quantitative factor assay
 - d. Stypven time test (Russell's viper venom time test)
4. Which test is prolonged in hypofibrinogenemia and affected by the levels of fibrinogen and dysfibrinogenemia, as well as the presence of heparin?
 - a. Thrombin time
 - b. Prothrombin time
 - c. Reptilase time
 - d. Fibrinogen
5. Which of the following conditions would show a short clotting time, indicating high fibrinogen levels?
 - a. DIC
 - b. Liver disease
 - c. Pregnancy
 - d. Fibrinolysis
6. Which condition would most likely show a decrease in factor VIII activity?
 - a. Hemophilia B
 - b. Liver disease
 - c. Myeloproliferative syndrome
 - d. von Willebrand's disease
7. Which aPTT result would indicate the presence of a strong circulating inhibitor?
 - a. aPTT not corrected by the addition of normal plasma
 - b. Normal aPTT that prolongs upon incubation at 37°C
 - c. aPTT corrected by the addition of normal plasma
 - d. Any of the above results
8. Which of the following are criteria for the laboratory detection of lupus anticoagulants?
 - a. Prolonged aPTT
 - b. No correction of aPTT after platelet neutralization procedure
 - c. Correction of aPTT after mixing studies
 - d. Negative ANA
9. Using the test to measure D-dimer, which test results would be indicative of a patient having DIC? (Assume the fibrinogen concentration is indicative of DIC.)
 - a. D-dimer test result of 800 ng/mL of D-dimer units; increased PT and aPTT; decreased platelet count
 - b. D-dimer test result of 800 ng/mL of D-dimer units; decreased PT and aPTT; increased platelet count
 - c. D-dimer test result of 230 ng/mL; increased PT, decreased aPTT; decreased platelet count
 - d. D-dimer test result of 230 ng/mL; decreased PT, increased aPTT; increased platelet count
10. Which of the following is a variable of the PFA-100 test?
 - a. Artery thickness
 - b. Puncture width
 - c. Temperature
 - d. Procedural standardization
11. Which statement is true of the PT and aPTT?
 - a. Together they assess all the factors in the coagulation system.
 - b. They are diagnostic tests for factor deficiency.
 - c. Neither are impacted by the administration of anticoagulant therapy.
 - d. Both provide excellent screening assays for factor deficiencies, the presence of inhibitors, and as monitoring assays for anticoagulant therapy.
12. Which factor assay utilizes the PT as its base testing mechanism?
 - a. Factor V
 - b. Factor VIII
 - c. Factor IX
 - d. Factor XII
13. The most common specific factor inhibitor is often present in patients who are:
 - a. Elderly
 - b. Hemophilic
 - c. Diagnosed with lupus erythematosus
 - d. Having spontaneous abortions
14. Which molecule functions in platelet adhesion and aggregation as a binding site for multiple coagulation components?
 - a. Protein C
 - b. Protein S
 - c. vWF
 - d. Antithrombin
15. Which of the following is a qualitative test for von Willebrand's disease?
 - a. vWF:Ag
 - b. vWF:RCo
 - c. vWF:Co
 - d. von Willebrand factor multimer assay
16. APC resistance is determined by testing for the presence of:
 - a. Antithrombin (AT-III)
 - b. Prothrombin G20210A mutation
 - c. FDPs
 - d. Factor V Leiden mutation (A506G)

REVIEW QUESTIONS—cont'd

17. Significantly increased quantities of coagulation activation molecular markers can indicate which condition?
 - a. Hemophilia A
 - b. Spontaneous thrombosis
 - c. Hemophilia B
 - d. Von Willebrand's disease
18. Which term describes an instrument that can maintain a constant temperature and has an automatically initiated timing device, but requires all reagents and samples to be delivered manually?
 - a. Fully automated
 - b. Semiautomated
 - c. Manual method
 - d. The tilt-tube method
19. Which is an example of clot formation detection measured by change in optical density?
 - a. Mechanical endpoint detection
 - b. Chromogenic endpoint detection
 - c. Photo-optical endpoint detection
 - d. Immunological endpoint detection

See answers at the back of this book.

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Applications of Flow Cytometry to Hematopathology

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CHAPTER OUTLINE

Basic Concepts of Flow

Cytometry
Threshold
Photodetectors
Amplification
Fluorescence Compensation

Flow Cytometric Analysis

Sample Preparation
Cytometer Operation
Data Analysis

Applications of Flow Cytometry

Lymphocyte Subset Analysis and CD4
T-Cell Enumeration
Leukemia and Lymphoma
Immunophenotyping
Leukemia and Lymphoma DNA
Content Analysis
Hematopoietic Progenitor Cell
Enumeration
Flow Crossmatching

Detection of Paroxysmal Nocturnal

Hemoglobinuria
Residual White Blood Cell Enumeration
Detection of Fetomaternal
Hemorrhage
Bead-Based Assays for Soluble Factors

Summary Chart

Case Study 34-1

Review Questions

References

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- 34-1 Analyze the role of light scatter in flow cytometry.
- 34-2 Describe the steps included in the sample preparation for flow cytometry testing.

- 34-3 Assess the basic principles of flow cytometer operation, including preparing samples, operating the instrument, data analysis, and result interpretation.
- 34-4 Evaluate the methods of flow cytometer data analysis.

Flow cytometry is a rapidly advancing technology that provides measurements (-metry) of physical characteristics of cells (cyto-) suspended in a moving fluid stream (flow). These measurements are made on a per-cell basis at rates of up to 10,000 cells per second. Because most hematologic tissues are easily prepared as cell suspensions, they are excellent candidates for flow cytometric analysis. The popularity of flow cytometry has been aided by the increasing availability of a large variety of monoclonal antibodies. These antibodies are used to mark cell lineages by detecting cell surface and intracellular proteins and can thereby determine cell lineage, identify cell differentiation, as well as monitor cell activation and other biological properties of clinical research-related significance. Innovation has produced smaller and increasingly user-friendly benchtop models of flow cytometers, which are becoming mainstream in larger laboratory facilities. This chapter presents basic concepts of flow cytometry and focuses on applications related to clinical hematology and hematopathology.

Basic Concepts of Flow Cytometry

Flow cytometers consist of three systems—the fluidics, optics, and electronics—in addition to a computer. The fluidics consists of pressurized air and saline sheath fluid that together carry the sample into the cytometer, through the flow cell, and

then to waste (Fig. 34-1). The sample flow rate and the cell concentration are adjusted so that, while in the flow cell, each cell passes through a laser beam, one at a time. For this, the flow cytometer uses a method called **hydrodynamic focusing**. The faster moving sheath fluid envelops the sample cell suspension and guides its particles single file through the narrow flow cell. If the pressure of the sample flow is increased, it is possible that more than one cell passes the **interrogation point** at a time, creating so-called coincident events that lead to false data. As a cell passes through the laser beam, multiple characteristics (parameters) are measured rapidly and simultaneously. The parameters measured are forward-scattered laser light (FSC), side-scattered laser light (SSC), and for clinical cytometers, multiple colors of fluorescent light. A four-color cytometer is depicted in Figure 34-1 with each detector designated FL1 through FL4 to denote each of the detectable colors. Flow cytometers may also use the fluorochrome name as the detector name (e.g., FITC detector, PE detector).

FSC and SSC parameters are based on the physical characteristics of a cell. The larger the cell, the greater the FSC signal, and the more internal complexity or granularity, the greater the SSC signal. The number of colors measured depends on the staining reagents used and whether or not the cell bound any reagents. The standard laser used in clinical systems is a 488-nm blue argon ion laser; therefore, the dyes

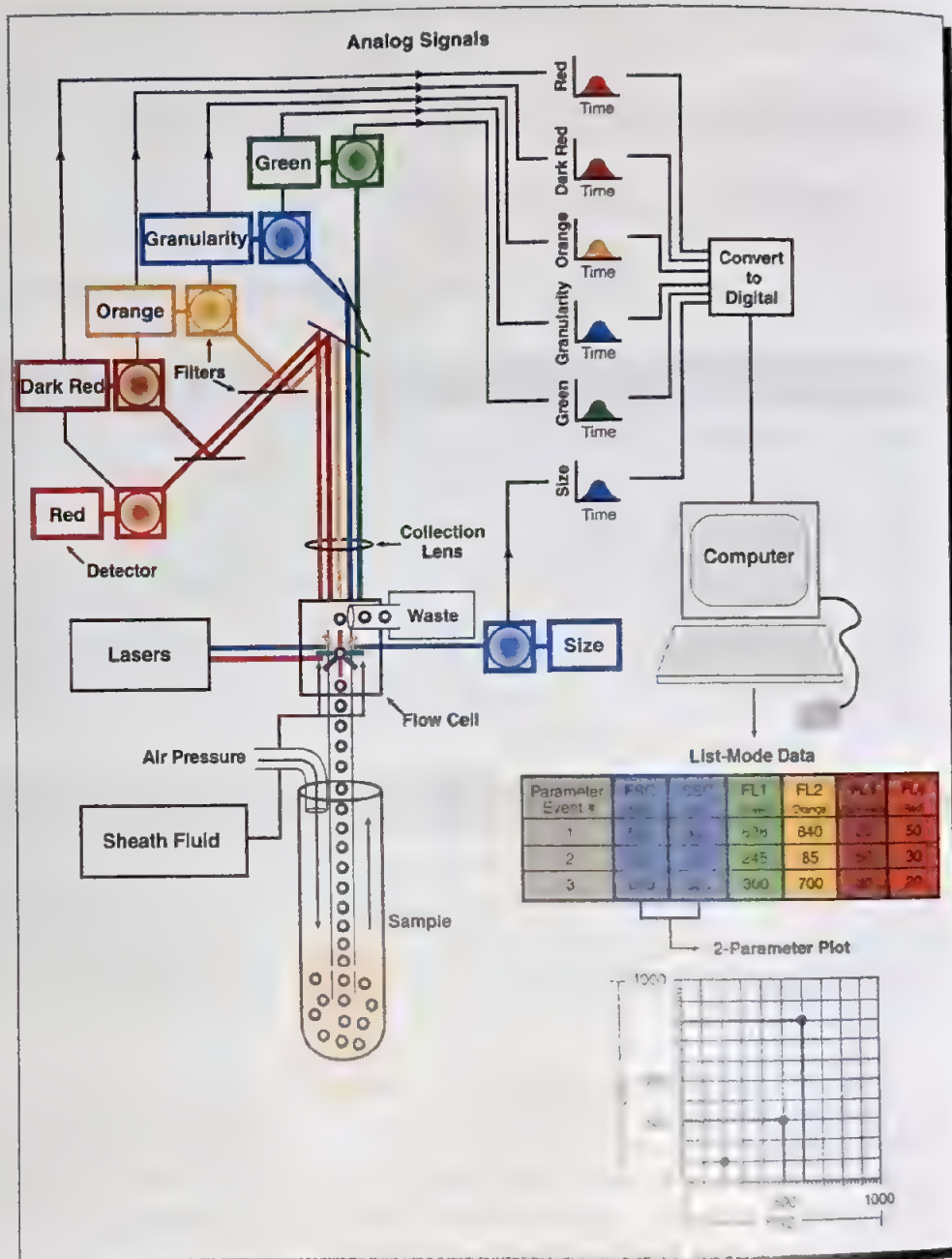


FIGURE 34-1 Schematic of cell analysis using a two-laser flow cytometer.

used must absorb or excite at this wavelength to produce fluorescent light. The dual laser system in Figure 34-1 includes a 635-nm red diode laser. Adding lasers expands the repertoire of fluorescent dyes that can be used. Research models typically have more laser options and are also capable of physically sorting out populations of interest for further studies.

As signals are emitted from the cell, the optical system separates and routes the different colored light to the appropriate photodetectors using strategically placed optical filters and mirrors. These mirrors and filters transmit, reflect, absorb, or block specific wavelengths of light. A dedicated photodetector measures the signal intensity for its respective parameter. In Figure 34-1, the optical system is set up to detect green (515 to 545 nm) signal in FL1/FITC, orange (564 to 606 nm) signal in FL2/PE, and two shades of red signals, one (greater than 670 nm) in FL3/PerCP and other (653 to 669 nm) in FL4/APC.

Threshold

Measurements are not made on every particle that passes through the laser beam. For a particle to be recognized by the system, it must first register above the threshold. The threshold parameter is used to limit what is analyzed by the cytometer. For example, if a sample contained numerous small particles (debris), the operator could eliminate them from the analysis by setting a threshold limit on size. In this instance, the cytometer assigns a particle an event number and displays its corresponding parameter values only if the particle's FSC signal is above the threshold limit. This ensures that the data will reflect measurements of cells rather than debris. Different applications utilize different threshold parameters and settings. For example, in leukocyte analysis assays, it is useful to add CD45 PerCP (a pan-leukocyte marker) to each sample and threshold on FL3 PerCP. Under these conditions, data are only collected on leukocytes because red-cell stroma and platelets are not "seen" by the cytometer.

The caveat here is to avoid setting the threshold too high because this may exclude a portion of the population of interest.

Photodetectors

Photodetectors convert the light signals into electrical signals called pulses. In Figure 34-1, the pulse shown for each parameter represents the signal intensity as the cell passes through the laser beam. The stronger the signal becomes, the higher the pulse. Electronic components measure the height of each pulse and eventually convert this information to a digital number.

The sensitivity of a detector can be increased by increasing its voltage. For example, the amount of voltage applied to the FL1/FITC detector depends on the sample. If the FITC signal is weak, as with a dimly staining population, the voltage must be increased to detect the population. If the signal is very bright and off scale, the voltage must be lowered. During optimization, the operator ensures that both bright and dim populations are on scale.

Amplification

For samples in which the signal range can be greater than 10-fold for a given parameter, logarithmic (log) amplification is used for that parameter. This allows that very bright and very dim signals can be displayed on the same scale.¹ Most cytometers use a four-decade log scale, meaning that the signals are converted to numbers between 1 and 10,000. Flow cytometers convert the digitized values to logarithms mathematically through the software.

Leukocyte immunophenotyping assays generally require the fluorescence data to be viewed in log and the scatter data to be viewed in linear because cells can exhibit very dim to very bright staining, whereas cell size and granularity measurements are confined to a smaller range. In contrast, for whole blood analyses of platelets and erythrocytes, log amplification of FSC is required to distinguish the two populations from one another.

Fluorescence Compensation

Many fluorescent dyes used in flow cytometry emit a wide array of wavelengths after absorbing laser light. The optical filters help ensure that the appropriate wavelengths reach the correct detector; however, some dyes such as PE, emit some green light, and this is detected by the FITC detector. Conversely, FITC emits a range of wavelengths, some of which are detected by the PE detector. This spillover into the "wrong" detectors also occurs with other dyes. Fluorescence compensation removes the effect of this spillover so that each detector reports only information for the appropriate fluorescent dye. While compensation is recommended as a daily routine, each laboratory may conduct their own studies to investigate the stability of compensation. It may be possible to stretch the time between compensation to up to a week, unless a change of antibody lots took place, which requires renewed compensation. Compensating due to a lot change is imperative for antibodies conjugated with so-called **tandem dyes**, for example, PE-Cy7. Besides being prone to losing fluorescent intensity over time due to extended light exposure or temperature changes, they also show more spillover variability from lot to lot.²

Tandem dyes are composites of two fluorochromes. Light excitation by the flow cytometer laser results in light emission from one fluorochrome, which, in turn, excites the other

fluorochrome that is attached. In the example of PE-CY7, PE is the donor fluorochrome that excites the CY7 receiver fluorochrome. In principle, in compensation a sample stained with only one fluorochrome is run and then adjustments are made so that the signal from the fluorochrome is only detected by its corresponding detector. This process is repeated for each fluorochrome used in the assay. Details for how these adjustments are made can be found elsewhere.^{1,3} Due to the complexity of compensating for increasingly high numbers of fluorochromes, the current generation of cytometers apply compensation mathematically to the digitized data through the software. Regardless of how compensation is performed, it must be set properly to ensure that the correct information is reported for each parameter. Methods for adjusting and checking compensation are provided by the cytometer manufacturers and are also available from other sources.³⁻⁵

Flow Cytometric Analysis

Flow cytometric analysis can be broken down into four main tasks: preparing samples, operating the cytometer, analyzing data, and interpreting results. These tasks may be performed by one to four individuals, depending on the organization of the laboratory and the expertise of the personnel. In the case of high-complexity leukemia and lymphoma phenotyping, the fourth task requires the expertise of a specialty physician. The next sections present general information about the tasks required to obtain flow cytometric results followed by assay-specific information.

Sample Preparation

Because flow cytometry measures cellular characteristics, it is important that the sample handling and preparation methods preserve these characteristics. The cellular characteristics measured are surface area, granularity and internal complexity, and fluorescent colors. The fluorescent colors usually result from reactions with staining reagents. Specimen collection factors that can affect cellular characteristics include the anticoagulant, storage temperature, and specimen age.

Specimen Collection and Handling

Specimen collection requirements vary based on the application. For example, for enumeration of leukocyte subpopulations, blood and marrow are usually collected in K₂ or K₃-ethylene diaminetetraacetic acid (EDTA), stored at room temperature, and analyzed within 24 hours of collection. In contrast, for assays of cell function, sodium heparin is usually the anticoagulant of choice. Thus, it is important that each laboratory verify conditions for specimen stability for each type of assay performed.

Lymphoid tissue specimens are also analyzed by flow cytometry. The tissue is harvested and placed in a sterile container containing cold tissue culture medium. Next, it is disaggregated into a single-cell suspension by gentle teasing with forceps, followed by filtration through a 50-micron (μ m) mesh. If needed, erythrocytes can be removed with lysing reagents such as ammonium chloride or via density gradients. Cell suspensions prepared from lymphoid tissues should be processed quickly to avoid artifacts caused by nonviable cells. A common density gradient medium used for blood, marrow, and lymphoid tissue

is Ficoll-Hypaque. After a volume of blood or cell suspension is underlayered with the medium and centrifuged, erythrocytes, dead cells, and myeloid cells pellet to the bottom of the tube while viable mononuclear cells remain at the gradient/plasma interface. When using gradient methods, it is important to verify that populations of interest are not also being removed.

Once the specimen is in single-cell suspension, aliquots are stained. Staining reagents provide additional information about a cell, and different reagents provide different pieces of information. For example, one reagent might bind in proportion to the amount of DNA present, whereas another might bind in proportion to the number of specific receptor sites present. Factors that can affect staining results include cell concentration of the aliquot, degree of saturation of cell receptors with stain, the temperature at which the staining is done, as well as the pH and tonicity of wash buffers. Accordingly, recommendations by the reagent manufacturers regarding staining conditions should be followed, and exposure of stained samples to direct light should be minimized.

Staining With Fluorescent Dyes

There are two approaches to staining cells. The first is to use special fluorescent dyes as staining reagents. These dyes have affinities for specific cell constituents. For example, propidium iodide (PI), 7-amino-actinomycin D (7AAD), and DRAQ5 bind to nucleic acids, while PKH-26 binds to membrane lipids. Some dyes, such as PI, require cell membrane permeabilization to reach its target, whereas other dyes, such as 7AAD and DRAQ5, can diffuse through the cell membrane. In addition, some dyes are equilibrium dyes, meaning that staining is measurable only when the cell is suspended in the dye, whereas for other dyes

staining can be observed only when excess dye is removed. A general staining scheme using fluorescent dyes is illustrated in Figure 34-2. Commonly used dyes are presented in Table 34-1.

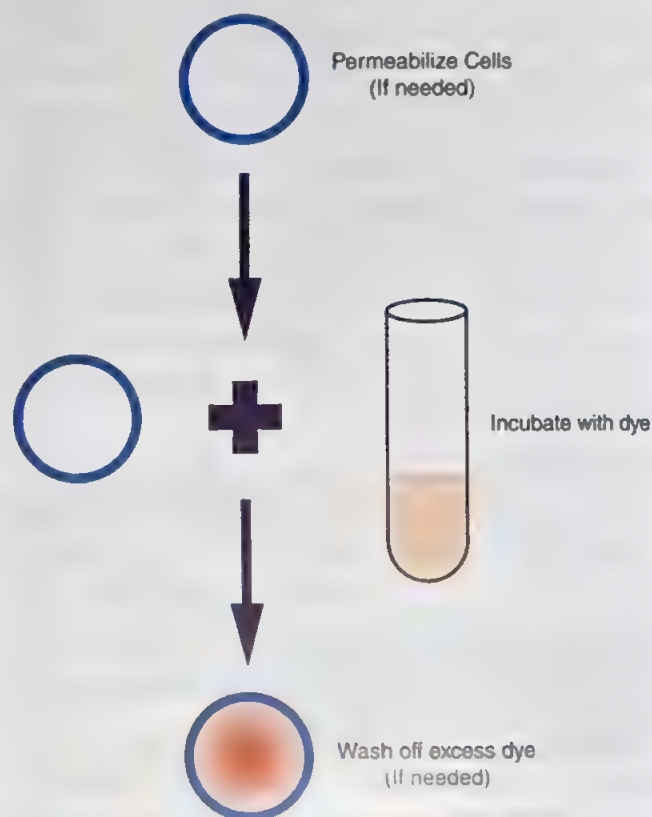


FIGURE 34-2 General staining scheme using fluorescent dyes.

TABLE 34-1 Common Fluorescent Dyes and Fluorochromes Used in Clinical Hematology

Application	Target	Excitation (nm)	Emission (nm)
DNA Content			
Propidium iodide (PI)	Double-stranded (DS) nucleic acids	488	575, 630
7-Amino-actinomycin D (7-AAD)	DNA	488	660
Acridine orange (AO)	DS nucleic acids	488	650
	Single-stranded nucleic acids	488	530
DRAQ5		488, 633	700
Reticulocytes			
Thiazole orange (TO)	RNA	488	525
Pyronin Y	RNA	488	575
Cell Physiology			
Fluo-3: cell activation	Ca ²⁺	488	525
SNARF-1: pH	H ⁺	488	575
DCFH-DA: oxidative burst	H ₂ O ₂	488	525
Rhodamine 123: drug efflux	Mitochondria	488	525
PKH-26: proliferation	Membrane lipids	488	525

TABLE 34-1 Common Fluorescent Dyes and Fluorochromes Used in Clinical Flow Cytometry—cont'd

Application	Target	Excitation (nm)	Emission (nm)
Immunophenotyping			
Fluorescein isothiocyanate (FITC)	Antigen (via antibody linked to fluorochrome)	488	525
Phycoerythrin (PE)	Antigen (via antibody linked to fluorochrome)	488	575
PE-Texas red (PE-TxR)	Antigen (via antibody linked to fluorochrome)	488	630
PE-carbocyanin-5 (PE-Cy5)	Antigen (via antibody linked to fluorochrome)	488	667
Peridinin-chlorophyll-protein (PerCP)	Antigen (via antibody linked to fluorochrome)	488	678
PerCP-Cy5.5	Antigen (via antibody linked to fluorochrome)	488	695
PE-Cy7	Antigen (via antibody linked to fluorochrome)	488	785
Allophycocyanin (APC)	Antigen (via antibody linked to fluorochrome)	633	660
APC-Cy7	Antigen (via antibody linked to fluorochrome)	633	785

Staining With Antibodies

In the second approach, the staining reagents are antibodies that have been chemically linked or conjugated to fluorescent dyes known as **fluorochromes**. This staining approach is called **immunophenotyping**. “Immuno-” refers to the use of antibodies and “phenotyping” refers to characterizing an organism or cell; therefore, immunophenotyping utilizes antibodies for characterizing cells. Only cells expressing the molecule or antigen recognized by the antibody will be stained with the fluorochrome. The antigen may be on the cell surface or intracellular.

Both polyclonal antibodies purified from antisera and monoclonal antibodies produced from cloned, immortalized B-cell cultures are used for immunophenotyping. Generally, monoclonal antibodies are preferred because they are homogeneous, highly specific, and well characterized. As shown in Table 34-1, several colors of fluorochromes are available for use with standard cytometers. Two important characteristics of fluorochromes are their absorption spectrum, at which they can be excited when passing the laser beam in the flow cell of the instrument, and their emission spectrum, which is the range of emitted wavelengths. The emission wavelength is longer than the excitation wavelength, and the difference between these wavelengths is called **Stoke's shift**. A high Stoke's shift is preferred because it gives greater separation between signal and noise.⁶ Table 34-1 lists the excitation and emission wavelengths for each fluorochrome. A general staining scheme for immunophenotyping leukocytes is illustrated in Figure 34-3.

Cells from different lineages or subpopulations bear unique combinations of antigens, and populations can be identified using appropriate antibody combinations. To do this, antibodies of different specificities are conjugated to different fluorochromes and then combined. The different antibodies are linked to fluorochromes that emit different colors, so the reactivity of each antibody can be tracked separately.

Standard antibody conjugates are commercially available for most routine applications. Extensive work goes into determining the optimal antibody concentrations, the appropriate fluorochrome pairing for each antibody in a test panel, and

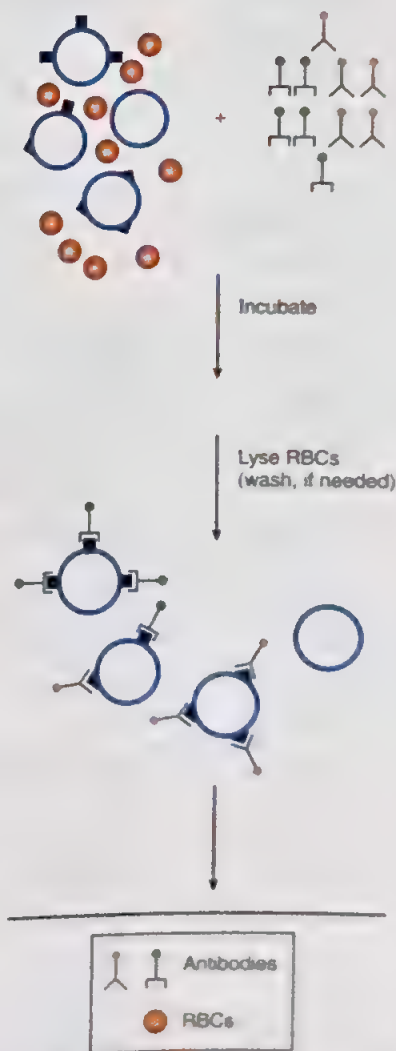


FIGURE 34-3 General staining scheme for staining leukocytes with antibodies. RBCs = red blood cells.

in validating the results obtained with the reagent. Commercially available antibody conjugates specific for a low-density antigen are typically paired with a bright fluorochrome such as PE, while an antibody specific for a high-density antigen is paired with an average intensity fluorochrome such as fluorescein isothiocyanate (FITC) or peridinin-chlorophyll-protein (PerCP). There are additional factors to consider when selecting antibody-fluorochrome combinations that are important when antibody test panels are validated by each laboratory as they are laboratory developed tests.

To provide a means for comparing monoclonal antibodies from different sources, a classification system has been devised.

The system identifies antibodies based on antigen reactivity, rather than by the name given by the manufacturer. With this classification, different antibodies binding the same antigen, as determined by flow cytometry and gel electrophoresis, are classified in the same "cluster of differentiation" or CD group. The CD groups are numbered, and antibodies within a group bind the same antigen. The International Workshop on Human Leukocyte Differentiation Antigens (HLDA) creates new CD groups when it meets periodically to evaluate and classify new antibodies and an updated list can be viewed at <http://www.hbdm.org>. The names and CD numbers of antibodies commonly used in hematopathology are listed in Table 34-2.

TABLE 34-2 Useful Antibodies for Hematology Applications

Applications								
Antibody	Antigen	Antigen	IM	AL	CLD	TL	PCD	Misc
CD1a	Gp49	Cortical and mature thymocytes, Langerhans' cells	-	(+)	-	(+)	-	
CD2	LFA-2 receptor	Thymocytes, NK cells, T cells	+	(+)	(+)	(+)	-	
CD3	T-cell receptor	Pan-T-cell marker	+	-	-	+	-	
CD4	MHC class II receptor	T-cell subset (helper T), monocytes, early myeloid progenitors	+	(+)	(-)	(+)	-	
CD5	Gp67	T cells, B-cell subset	-	(+)	-	-	-	
CD7	Gp40	T cells, NK-cell subset, progenitor cell subset	-	-	(+)	(-)	-	
CD8	MHC class I receptor	T-cell subset (cytotoxic T), NK-cell subset	+	(+)	(+)	(+)	-	
CD10	CALLA, neutral endopeptidase	Pro- and pre-B cells, granulocytes	-	-	-	-	-	
CD11b	α M integrin chain, CD33aR, CP3	Granulocytes, monocytes, NK cells, T- and B-cell subsets	-	-	-	-	-	
CD11c	p150, 95	Neutrophils, monocytes, NK cells	-	-	-	-	-	
CD13	Aminopeptidase N	Granulocytes, monocytes	-	-	-	-	-	
CD14	LPS receptor	Monocytes, macrophages, neutrophils	-	-	-	-	-	
CD15	Lewis' X hapten	Granulocytes, monocytes, progenitor cell subset, mast cells	-	-	-	-	-	PNH
CD16	Fc γ RIII receptor	Granulocytes, NK cells, monocytes	-	-	-	-	-	PNH
CD19	Gp45	Pan-B-cell marker	-	-	-	-	-	
CD20	p35	B cells	-	-	-	-	-	
CD22	BL-CAM	B cells	-	-	-	-	-	
CD23	Fc ϵ receptor	Eosinophils, B cell subset	-	-	-	-	-	
CD25	IL-2 receptor	T cells	-	-	-	-	-	
CD30	Ber-H2 antigen, Ki-1 antigen	Activated lymphocytes, monocytes, Reed-Sternberg cells	-	-	-	-	-	
CD32	Fc γ RII receptor	Granulocytes, monocytes, B cell subset	-	-	-	-	-	
CD33	Gp67	Monocytes	-	-	-	-	-	
CD34	Gp116	Progenitor cells	-	-	-	-	-	PCE
CD36	GPIIb, GPIV, OKM5 antigen	Platelets, mature monocytes, macrophages, microvascular endothelial cells, dendritic cell subset	-	-	-	-	-	

TABLE 34-2 Useful Antibodies for Hematology Applications—cont'd

Applications		Antigen	Antigen	IM	AL	CLD	TL	PCD	Misc
CD38	ADP-ribosyl cyclase	Subsets in all hematopoietic lineages		-	(+)	(+)	(+)	+	
CD41	GPIIb	Megakaryocytic cells, platelets		-	(+)	-	-	-	
CD42b	GPIIb α	Megakaryocytic cells, platelets		-	(+)	-	-	-	
CD45	LCA	All leukocytes		+	+	+	+	+	PNH, PCE
CD55	Decay-accelerating factor	Erythrocytes, granulocytes			-		-	-	PNH
CD56	N-CAM	T-cell subset, NK cells		+	(+)	(+)	(+)	+	
CD57	Oligosaccharide	T-cell subset, NK-cell subset		-	-	-	-	-	
CD59	Membrane inhibitor of reactive lysis	Erythrocytes, granulocytes			-	-	-	-	PNH
CD61	GPIIb	Megakaryocytic cells, platelets		-	(+)	-	-	-	
CD64	Fc γ R1 receptor	Myelomonocytic cells, activated neutrophils		-	(+)	-	-	-	
CD65	Ceramide 12-saccharide	—		-	-	-	-	-	
CD71	Transferrin receptor	Upregulated on proliferating cells		-	(+)	-	(+)	-	
CD79a	Ig α ; MB1, B antigen receptor subunit	B cells		-	(+)	-	-	-	
CD79b	B29; Ig β ; B antigen receptor subunit	B cells		-	-	(+)	-	-	
CD103	α E integrin subunit	T cells, monocytes, hairy-cell leukemia		-	-	(+)	-	-	
CD117	c-kit, stem cell factor receptor	Stem cells		-	(+)	-	-	-	
CD138	Heparan sulfate proteoglycan syndecan-1					-	-	+	
CD235a	Glycophorin A	Erythroid cells			(+)	-	-	-	
Bd 2				-	(+)		-		
FMC7		B cells		-	-	(+)	(+)	-	
HgbF	Hemoglobin F	Fetal erythrocytes				-	-	-	FMH
HLA DR	MHC class II	T-cell subsets, monocytes, all B cells, NK cells, progenitor cells		(+)	-	-	-		
IgM/D/A	Ig heavy chains	B cells		(+)	(+)	(+)	+		
Kappa/Lambda	Ig light chains	B-cell subset, all cells with Fc receptors		+	+	+	+		
TCR $\alpha\beta$ / $\gamma\delta$	T-cell receptor	T cells		-	-	(+)	(+)	-	
TdT	Terminal deoxynucleotidyl transferase	Pro- and pre-B cells, cortical thymocytes		(+)	-	(+)	-		
MPO	Myeloperoxidase	Myeloid cells		(+)	-	-	-		

AL = acute leukemia; IM = immune monitoring; CLD = chronic lymphoproliferative disease; TL = tissue lymphoma; PCD = plasma cell disorders; NK = natural killer; PNH = paroxysmal nocturnal hemoglobinuria; PCE = progenitor cell enumeration; FMH = fetomaternal hemorrhage; + = essential; (+) = useful; - = useful for some cases. Sources: Antibody data compiled from references 14 and 15.

ADVANCED CONTENT

Antibody titration in flow cytometry is the process of identifying the optimal concentration of antibody for a given assay. When selecting antibody fluorochromes, it is necessary to determine the volume of antibody to be added to the sample cell suspension, since sensitivity and reactivity are affected by the antibody concentration.⁸ If a sample cell suspension receives an excess of conjugate, background interference may be caused by nonspecific binding. Conversely, if too little antibody is used, it decreases the positive signal and may result in false-negative interpretation. Commercial antibody conjugates generally carry manufacturer recommended volumes, which should be used as the starting point for serial dilutions during titration. For example, the manufacturer may recommend 20 μL of the conjugate per 1 million cells in 100 μL . It is important to perform the titration by altering the amounts of conjugate but keeping cell concentration and total reaction volume constant. Table 34-3 shows antibody dilutions based on 20 μL manufacturer recommendation. When selecting the cell suspension for this experiment, both cell types that carry the target antigen and those that do not are included. After data acquisition the **Staining Index (SI)** is calculated. This index demonstrates the optimum separation between an antigen-negative and antigen-positive population and can be displayed by graphing antibody volume against the SI value. Figure 34-4 shows the optimal concentration at the

TABLE 34-3 Antibody Dilutions Based on 20 μL Manufacturer Recommendation

Method	Dilutions Starting Volume: 20 μL			
Titration	Undiluted	1:2	1:4	1:8
Antibody	20	10	5	5
PBS	0	0	0	5
Final Amount	20	10	5	2.5

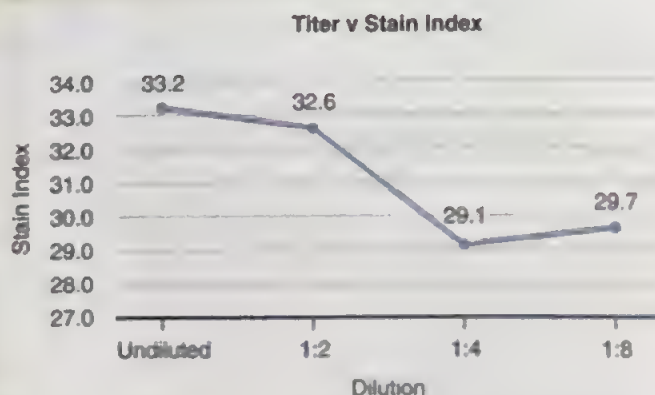


FIGURE 34-4 Graph showing the Stain Index (SI) for each antibody conjugate dilution.

highest point of the line. In this example, 20 μL , the volume recommended by the manufacturer, was confirmed to be the ideal amount of antibody conjugate to be added to the sample.

Cytometer Operation

Five procedures are required for cytometer operation: startup, quality control, optimization, data collection, and shutdown.

Startup

Each cytometer manufacturer provides a startup procedure. Startup includes turning on the cytometer and computer, filling sheath and emptying waste reservoirs, and priming fluidics.

Quality Control

Quality control (QC) procedures are performed to monitor instrument performance and to ensure consistency from day to day. For this purpose, the manufacturers provide a QC software program that is used in conjunction with fluorescent beads. The beads are run under standard conditions, and measurements are made. Because the beads are very stable, any major fluctuations in these measurements indicate a possible cytometer problem. Additional QC procedures may be required depending on the application.^{4,5}

Optimization

During optimization, the operator adjusts the cytometer settings so that cell populations in the sample can be properly analyzed. This is analogous to what is done to prepare for examining a specimen on a microscope slide. Once the slide is put on the stage, the lighting, optics, and stage position are adjusted so that the cells can be seen. Similarly, optimization entails running a sample from a normal donor and adjusting the cytometer settings to bring the populations of interest on scale. It is important to use either cells from a healthy subject or commercially prepared cell controls with known characteristics, because patient samples may lack normal populations. Cytometers that use beads for daily quality control may only have to be optimized when a baseline for these beads is established every 6 months. Consequently, as long as the daily QC provides acceptable results, optimization continues to be valid. To understand which settings can be adjusted, it helps to understand how a cytometer functions. The next section combines an overview of cytometer function with explanations of the instrument settings that can be adjusted during optimization.

Data Collection

After optimization is complete, the operator sets up the software to collect and store data files for each sample. The operator inputs information such as the number of events to collect, the file name, the patient identification number, and the staining reagents used for each sample. As the sample is run, the parameter measurements for each event are stored in a list that continues to grow until the number of events to be analyzed has been reached. The resulting data file contains

the parameter data along with information entered by the operator. A data file containing information for three events is depicted in Figure 34-1, but typical files contain information for at least 10,000 events.

Shutdown

A shutdown procedure is provided by the cytometer manufacturer. It consists of cleaning and decontaminating the cytometer and then turning the power off. Depending on the dyes used for an assay, it may be necessary to perform a short cleaning procedure between assays. This avoids possible dye carryover to subsequent samples. If the flow cytometer is shut off for long time periods, it is important to not let sheath fluid remain in the lines, as this can lead to salt crystal build-up. In laboratories that do not operate 24 hours a day, it is necessary to fill the cytometer lines with a specially formulated shutdown solution overnight that is then replaced again by sheath solution upon startup.

CRITICAL THINKING QUESTION

34-1 When preparing the settings to collect data, why is it important to pay close attention to the rate at which the sample passes through the flow cell?

See answers to all Critical Thinking Questions at the back of this book.

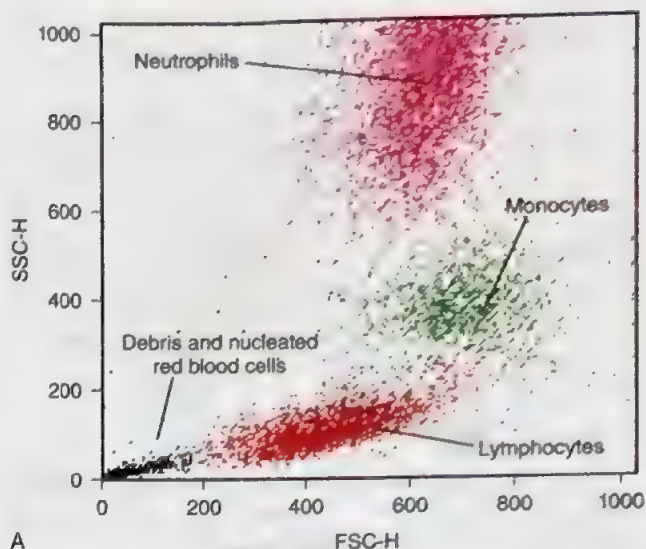
Data Analysis

There are basically two objectives for performing data analysis. One is to obtain statistics. This is because population percentages, absolute cell counts, and fluorescence intensity measurements are typically reported. The other objective is to identify abnormal populations based on light scatter, staining patterns, or both. The prerequisite for performing this type of analysis, of course, is to be able to identify normal populations using the same parameters.

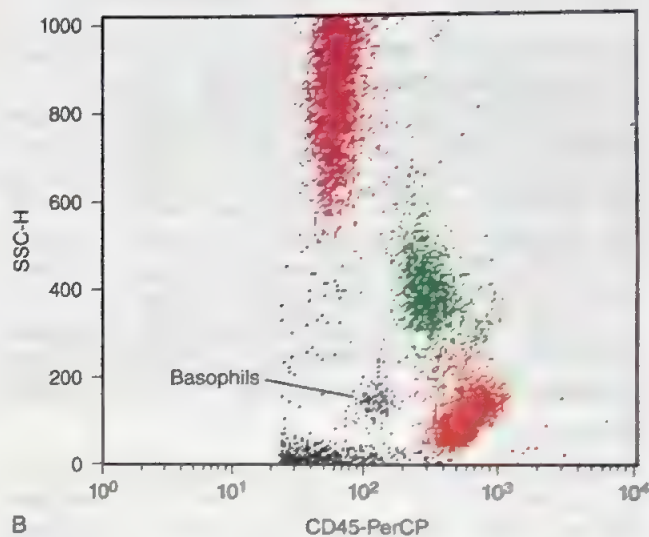
Identifying Populations

Because most hematologic samples contain multiple cell populations, it is necessary to first identify the population of interest for further analysis by visual inspection of the data in a plot. The three dots displayed in the FSC/SSC plot represent the three events in the data file (see Fig. 34-1). The x and y coordinates of each dot represent the event's FSC and SSC measurements; therefore, the location of each dot is very informative. With only three events, however, it is impossible to determine whether multiple populations exist.

A similar plot is shown in Figure 34-5A with 10,000 events displayed. These data were collected from a blood sample that was stained and then lysed to remove erythrocytes. With this large data set, it is possible to visualize multiple populations. Three cell populations are displayed and color-coded for clarity. Lymphocytes are small and contain no granules; therefore, the FSC and SSC values are low compared with those for the monocytes and neutrophils. Monocytes are larger and contain more vacuoles than lymphocytes; as a result, the FSC and SSC values are higher. Finally, neutrophils contain many granules, yet they are similar in size to monocytes; therefore, their FSC values are similar but their SSC values are much higher than monocytes.



A



B

FIGURE 34-5 Leukocyte populations discernible in lysed whole blood. **A.** FSC/SSC display. **B.** CD45/SSC display. Note how in **B**, debris and basophils can be resolved from the lymphocyte population.

In Figure 34-5B, the same file is displayed with CD45-PerCP on the x-axis rather than FSC. This plot illustrates the utility of CD45 for resolving leukocyte populations. Using the CD45/SSC plot, it is now possible to resolve debris, nucleated red blood cells, and basophils from lymphocytes. Although the FSC/SSC plots are useful, CD45/SSC plots are being used more frequently for leukocyte analysis, especially for bone marrow samples in which interfering nucleated red cells are more frequent.

Whole blood is commonly used for platelet and erythrocyte assays. The location of platelet, erythrocyte, and leukocyte populations in whole blood is displayed in Figure 34-6. For some applications, other parameter combinations are used for identifying populations. They are discussed where relevant.

Gating

Many analyses focus on a single population. Gating is a software feature used to restrict analysis to a particular

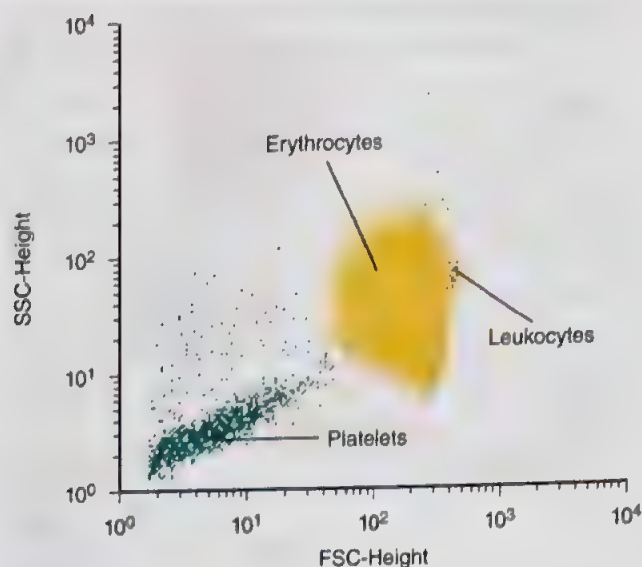


FIGURE 34-6 Populations discernible in whole blood.

population. A gate is created by drawing a graphical boundary around a population.

In Figure 34-7A, a gate has been drawn around the lymphocytes. The effect of using no gate or using the lymphocyte gate to display the FITC and APC fluorescence values from the data file is compared in Figures 34-7B and 34-7C. With no gate, the fluorescence of monocytes, neutrophils, and debris is displayed along with the lymphocyte fluorescence, making it impossible to obtain statistics on lymphocytes alone. The lymphocyte gate restricts the fluorescence data, making statistical calculations possible for a particular population. Next, we will see how these calculations are done.

Quadrant Statistics

Quadrant markers divide two-parameter plots into four sections called *quadrants*. The quadrants are used to distinguish negative, single-positive, and double-positive populations from one another. Using Figure 34-7C as an example, negative events are represented by dots with low FITC and APC values, single-positive events are represented by dots with either high FITC and low APC values or low FITC and high APC values, and double-positive events are represented by dots with high FITC and APC values.

Quadrant markers are set to encompass the negative population within the lower left (LL) quadrant (Fig. 34-7C). For applications in which it is difficult to distinguish the negative population from the single-positive populations, a replicate sample stained with a subclass control reagent is used. This control reagent contains an antibody of the same immunoglobulin subclass as the staining reagent, conjugated to the same fluorochrome, but specific for an antigen not found in humans. These staining conditions produce a negative population appropriate for setting quadrants.

Percentages are calculated based on the number of events within each quadrant. The statistics for Figure 34-7C are shown in Figure 34-8.

Events falling in the upper right (UR) quadrant are double-positive and, according to the statistics, this quadrant contains

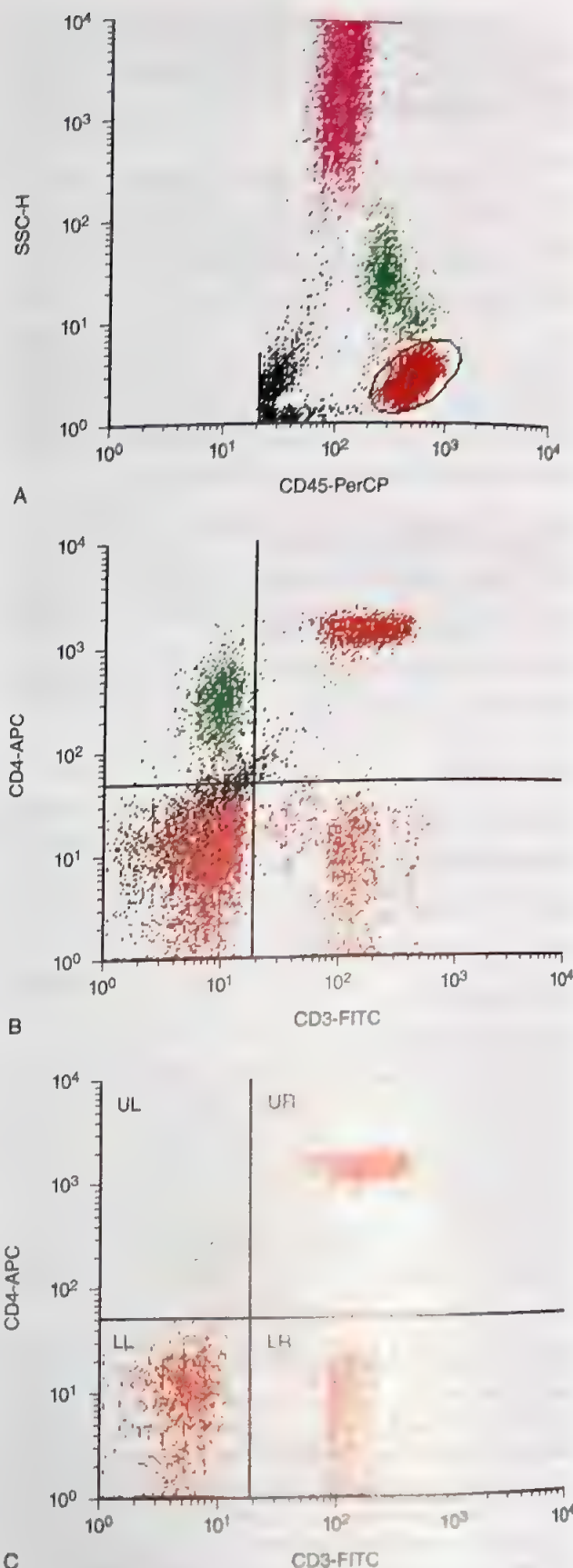


FIGURE 34-7 Effect of a lymphocyte gate (A) on fluorescence. Fluorescence events in B (ungated) and C (gated data) are color coded to their respective populations in A; using a gate provides a means for analyzing one population at a time. UL = upper left; UR = upper right; LL = lower left; LR = lower right.

Gated Events: 3484
Total Events: 10000

Quad	Events	% Gated	% Total
UL	72	2.07	0.72
UR	1184	33.98	11.84
LL	1321	37.92	13.21
LR	907	26.03	9.07

FIGURE 34-8 Quadrant statistics from Figure 34-7C. UL = upper left; UR = upper right; LL = lower left; LR = lower right.

1,184 events. The % Gated statistic reflects the percentage of lymphocytes that are double-positive. It was calculated by dividing the number of events in UR by the number of events in the lymphocyte gate $[(1,184 \div 3,484) \times 100 = 33.98]$. To calculate the events in UR as a percentage of the total events (% Total), the total event number is used as the denominator $[(1,184 \div 10,000) \times 100 = 11.84]$. The % Gated statistics are used most frequently because they are based on the population of interest.

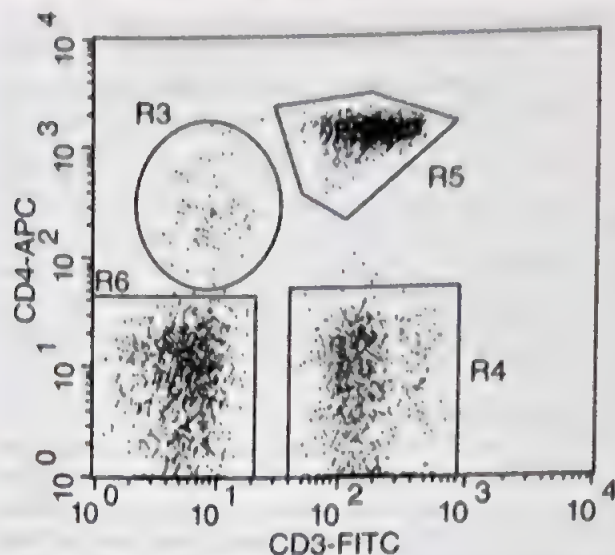
Region Statistics

An analysis tool to separate distinct cell populations are regions. Four regions are displayed in Figure 34-9. In contrast to quadrants, regions can be drawn in various shapes to fit each population. The % Gated and % Total statistics are calculated as in quadrant statistics, except that the number of events in each region is used as the numerator. Figure 34-9 displays the same data shown in Figure 34-7C; thus, the region R5 statistics are comparable to the UR statistics in Figure 34-7. In region analysis, the operator usually readjusts the regions for each donor even though the samples are stained with the same reagents. This is necessary because populations can shift from one donor to another.

A variation of region analysis uses movable regions linked to cluster-seeking algorithms. The algorithm directs the region to home to its target population as each new data file is analyzed.

Single-Parameter Histogram Statistics

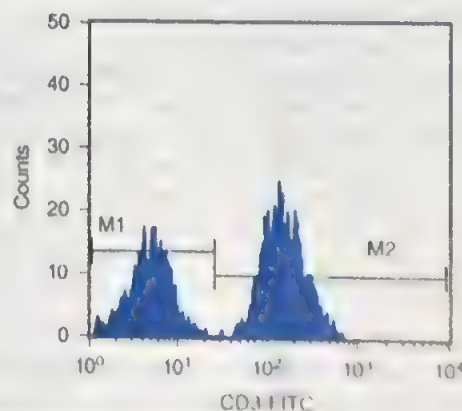
For some applications, statistical information from one parameter is sufficient. Figure 34-10 displays the FITC data shown in Figure 34-9. The FITC values are displayed on the x-axis, and the number of events expressing each value is displayed on the y-axis. Markers are set so that events with FITC values higher than those of the negative population can be measured. As with quadrants, if the negative population is difficult to distinguish from the positive population, a subclass control sample can be used to determine where to set the markers. Percentages are calculated using the number of events between the markers as the numerator and the gated events or total events for the denominator. In this example, 59.87% of the lymphocytes were positive for CD3.



Gated Events: 3484
Total Events: 10000

Region	Events	% Gated	% Total
R2	3484	100.00	34.84
R1	62	1.78	0.62
R3	68	1.95	0.68
R4	908	26.06	9.08
R5	1171	33.61	11.71
R6	1322	37.94	13.22

FIGURE 34-9 Analysis using regions.



Gated events: 3484
Total events: 10000

Marker	Left, Right	Events	% Gated	% Total	Median
All	1, 9910	3484	100.00	34.84	95.17
M1	1, 26	1384	39.72	13.84	5.09
M2	26, 9140	2086	59.87	20.86	148.55

FIGURE 34-10 Analysis using a single-parameter histogram with markers.

Histograms displaying cellular DNA content measurements (Fig. 34-11) require the use of specialized algorithms for calculating population percentages. In DNA analysis, cells are stained with a fluorescent dye that binds in proportion to the amount of DNA present. Analysis of proliferating cells stained with DNA dyes reveals three populations: those with the normal complement of DNA, those with a double complement of DNA, and those in between. These populations are referred to as G_0/G_1 -phase cells, $G_2 + M$ -phase cells, and S-phase cells, respectively. The location of these populations and the percentages, as computed using a DNA analysis program, are illustrated in Figure 34-11.

Absolute Cell Counts

So far, methods for obtaining percentage statistics have been presented. In many applications the absolute count, which is reported as the number of cells per microliter of specimen, is more useful. In flow cytometry there are two ways to obtain this statistic without relying on hematology analyzer information. The first requires that the cytometer analyze a fixed volume of sample. That way, the number of events for the population of interest can be expressed per microliter of sample, and this can be extrapolated back to the volume of specimen used to prepare the sample. For example, if there are 3,000 events of the population of interest in 20 μL of sample, and the sample contains 1:10 dilution of whole blood, then the absolute count is calculated as:

$$\frac{3,000 \text{ events}}{20 \mu\text{L sample}} \times 10 = 1,500 \text{ events}/\mu\text{L blood}$$

Because few cytometer models can measure fixed volumes of sample, the more common way to obtain absolute counts

utilizes fluorescent reference beads. In this approach, a known number of reference beads is added to the sample, which also contains a known volume of specimen. A data file is collected, and region statistics are used to measure the number of cell population events and the number of bead events. The ratio of these two numbers is determined and the absolute count is calculated using the formula:

$$\frac{\text{No. cell population events}}{\text{No. bead events}} \times \frac{\text{No. beads per sample}}{\mu\text{L specimen per sample}} = \text{No. cells}/\mu\text{L specimen}$$

Fluorescence Intensity Measurements

For some applications, population percentages and absolute counts are not useful. Instead, the amount of staining reagent bound is informative, so the fluorescence intensity is measured. This is sometimes referred to as the mean (or median) fluorescent intensity or MFI. The median is most commonly reported for this purpose because it is less affected by bright outliers, and it best represents the fluorescence per cell in a population. Reviewing the statistics in Figure 34-10, the median fluorescence of the M2 peak is 148.6. One can also make relative comparisons of antigen expression using this statistic. For example, since the staining reagent is an antibody, then the fluorescence intensity is related to the number of antigens present. For the data in Figure 34-10, a ratio of the medians of the M2 and M1 populations (148.55/5.09) indicates that positive cells (M2) bear approximately 30 times more antigen than the negative cells (M1). This is only a relative measurement, however, and the actual number of antigen molecules present cannot be quantitated without additional information. Mean fluorescence intensity is a useful measurement when performing lot-to-lot comparison studies of antibody conjugates as part of quality assurance. The % difference between the MFI of the old lot and the new lot of antibody can be used for an acceptance criterion to ascertain consistency in patient testing.

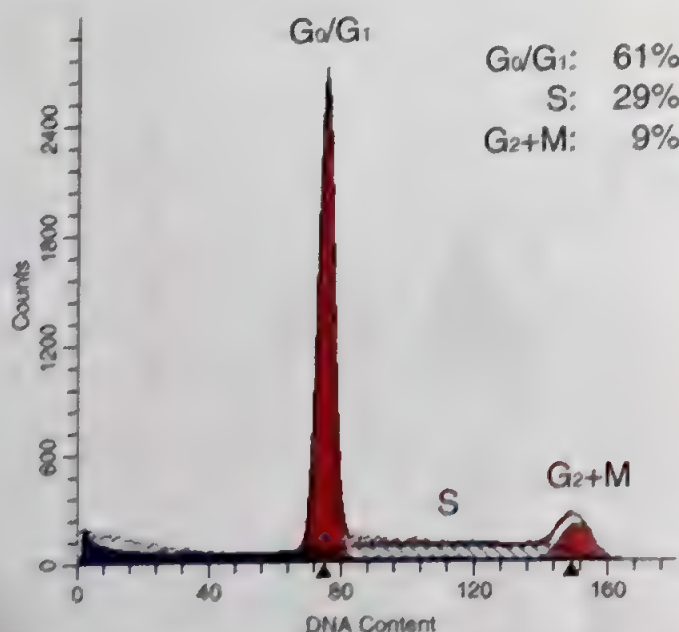


FIGURE 34-11 Analysis of DNA data using a single-parameter histogram and software modeling.

CRITICAL THINKING

34-2 A laboratory policy may state that, if a new antibody lot is 15% dimmer than the current lot, the new lot may have to be replaced by the manufacturer. However, if the new lot is >20% brighter than the current lot, the new lot should not be tested. Before putting the new lot into use, what action is necessary to assure consistency in patient testing?

Applications of Flow Cytometry

In clinical flow cytometry, methods for determining lymphocyte subsets and for analyzing leukemias and lymphomas are common. Visit www.tadacell.com and register your access code to see the required steps for the following procedures.

Lymphocyte Subset Analysis and CD4 T-Cell Enumeration

Lymphocyte subset analysis and CD4 T-cell enumeration assays are used to evaluate immunodeficiency disease states. The subsets measured are the T-cytotoxic, T-helper, B, and natural killer (NK) lymphocytes. For more details on this application and the required steps for this procedure, visit www.fadavis.com.

Leukemia and Lymphoma Immunophenotyping

Leukemia and lymphoma immunophenotyping can be used to detect, characterize, and monitor abnormal cell populations. Leukemia is a term used to denote a malignancy that occurs at any step in the differentiation from a blast in the bone marrow to a mature leukocyte in the peripheral blood. Leukemia may be lymphoid or myeloid in origin and may be classified as acute or chronic depending on the onset. *Lymphomas* are malignancies of the lymphoid system. For more details on this application and the required steps for this procedure, visit www.fadavis.com.

Leukemia and Lymphoma DNA Content Analysis

Leukemia and lymphoma DNA content analysis provides an additional way to characterize malignant cells. In contrast to immunophenotyping, which can provide diagnostic information, DNA content analysis mainly provides prognostic information. The percentages of cells in each phase of the cell cycle are estimated based on the amount of DNA present in each cell. For more details on this application and the required steps for this procedure, visit www.fadavis.com.

Hematopoietic Progenitor Cell Enumeration

Hematopoietic progenitor cells (HPC), sometimes called hematopoietic stem cells, are CD34-positive leukocytes found primarily in bone marrow and cord blood, and in low numbers (1%) in the peripheral blood. They can replace failed marrow in various anemias and are being used to rescue cancer patients from the fatal aplasia that results from high-dose chemotherapy.⁹ In the past, bone marrow samples were harvested from the patient before chemotherapy or from a human leukocyte antigen (HLA)-matched donor. This method was tedious and painful for the donor. With the advent of HPC mobilization techniques, it has become possible to obtain large numbers of peripheral blood progenitor cells through apheresis. For more details on this application and the required steps for this procedure, visit www.fadavis.com.

Flow Crossmatching

Flow crossmatching is used to determine whether a recipient contains alloantibodies against cells from a potential donor. The test assists in identifying transplantation recipients with a high risk for rejection, and it can also aid in monitoring for rejection post-transplantation. Numerous

studies have shown that even low concentrations of alloantibodies can be associated with engraftment failure. For more details on this application and the required steps for this procedure, visit www.fadavis.com.

Detection of Paroxysmal Nocturnal Hemoglobinuria

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare acquired clonal disorder of hematopoietic stem cells caused by a somatic mutation of the X-linked *PIG-A* gene, which is involved in the synthesis of glycosyl phosphatidyl inositol (GPI), an anchor molecule that attaches proteins to the cell membrane.¹⁰ This leads to a diminished expression of GPI-linked proteins, two of which are responsible for the susceptibility of erythrocytes to lysis by complement. They are CD55 and CD59, decay-accelerating factor (DAF) and membrane inhibitor of reactive lysis, respectively. For more details on this application and the required steps for this procedure, visit www.fadavis.com.

Residual White Blood Cell Enumeration

Flow cytometry can be used for enumerating residual white blood cells in leukocyte-reduced blood products. Leukocytes in blood products for transfusion can cause adverse effects in patients, such as nonhemolytic febrile reactions, alloimmunization, or transmission of infectious agents. For more details on this application and the required steps for this procedure, visit www.fadavis.com.

Detection of Fetomaternal Hemorrhage

The detection of circulating fetal erythrocytes in maternal blood is key for the obstetric management of blood group Rh-negative women carrying a Rh-positive fetus and for assessing placental injury after trauma. Through flow cytometry, it is possible to detect as low as 0.02% fetal erythrocytes in maternal blood, making this assay a type of rare event analysis.¹¹ For more details on this application and the required steps for this procedure, visit www.fadavis.com.

A variety of flow cytometry assays exist for platelet studies. They include assays to measure platelet activation status, platelet activation function, platelet-bound immunoglobulin, and platelet production. This information is useful for evaluating patients with coronary artery disease to determine whether anticoagulant therapy is sufficient, for studying congenital platelet disorders, and for evaluating thrombocytopenias.^{12,13} For more details on this application and the required steps for this procedure, visit www.fadavis.com.

Bead-Based Assays for Soluble Factors

The utility of flow cytometry is expanding the detection of soluble factors. These assays use sandwich techniques whereby a capture molecule, usually an antibody, is attached to the surface of beads. For more details on this application and the required steps for this procedure, visit www.fadavis.com.

SUMMARY CHART

- The cellular characteristics measured in flow cytometry include surface area, granularity and internal complexity, and fluorescent colors.
- Immunophenotyping utilizes fluorochrome-conjugated antibodies to identify target populations.
- Antibodies within a CD (cluster of differentiation) group bind the same antigen or epitope.
- The objectives of data analysis in flow cytometry include obtaining statistics on population percentages, absolute cell counts, and fluorescence intensity measurements; identifying abnormal populations based on light scatter or staining patterns; or both.
- Gating is a software feature that allows the operator to focus on a single population of cells.
- Quadrant statistics are used to distinguish negative, single-positive, and double-positive cell populations

CASE STUDY 34-1 Diagnosis of Chronic Lymphocytic Leukemia by Flow Cytometry

REASON FOR VISIT A 68-year-old male patient arrived in the emergency department with symptoms of a heart attack. As part of his initial laboratory orders, a complete blood count with differential was performed.

PATIENT AND FAMILY MEDICAL HISTORY

The patient remarked that he had not seen his primary care physician in 3 years. However, he had been feeling more tired during the last few months.

MEDICATION HISTORY OTC Aspirin, 1/Day

PHYSICAL FINDINGS Exam showed palpable cervical lymph nodes and mild hepatomegaly.

INITIAL LAB RESULTS

Automated CBC and Differential

Laboratory Test	Result	Units
RBC	2.17	$\times 10^6/\mu\text{L}$
Hb	6.6	g/dL
Hct	23.1	%
MCV	106.4	fL
MCH	30.4	pg
MCHC	28.6	g/dL
PLT	90.8	$\times 10^3/\mu\text{L}$
WBC	277.8	$\times 10^3/\mu\text{L}$
Neutrophil %	1	%
Abs. Neutrophils	2.8	$\times 10^3/\mu\text{L}$
Lymphocyte %	99	%
Abs. Lymphocytes	75.0	$\times 10^3/\mu\text{L}$

QUESTIONS

1. Considering the automated CBC results, what is the patient's differential diagnosis?
2. What additional step is helpful in the differentiation?

ANSWERS

1. The patient has a markedly elevated white blood cell count and absolute lymphocytosis. Hemoglobin and platelet count are also reduced.
2. A peripheral blood smear must be reviewed to evaluate cell features.

SMEAR REVIEW OBSERVATIONS Generally, mature lymphocytes display nuclei with condensed chromatin and "soccer-ball"-like appearance. No blast cells, but many smudge cells, are noted. An albuminized smear additionally reveals occasional prolymphocytes.

QUESTIONS

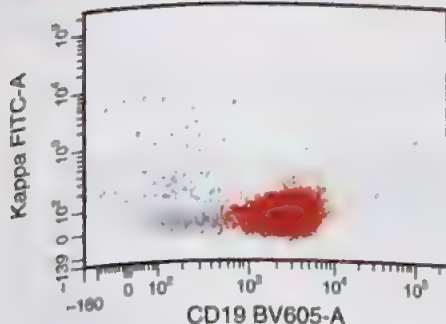
3. What condition is most likely indicated by the observations during the peripheral blood smear review?
4. What follow-up testing is indicated to make a diagnosis?

ANSWERS

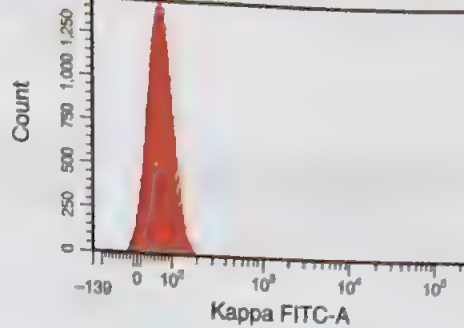
3. Neither blasts nor reactive lymphocytes were noted, ruling out acute leukemia and a reactive process due to infection. Given the prevalence of mature lymphocytes with condensed chromatin (soccer ball appearance) and the presence of smudge cells and prolymphocytes on the albuminized smear are indicators of possible chronic lymphocytic leukemia (CLL).
4. Flow cytometry testing on the peripheral blood is indicated to rule out or diagnose CLL. The peripheral blood specimen was taken to the flow cytometry lab and tested. These are the scatter plots (next page).

CASE STUDY 34-1 Diagnosis of Chronic Lymphocytic Leukemia by Flow Cytometry—cont'd

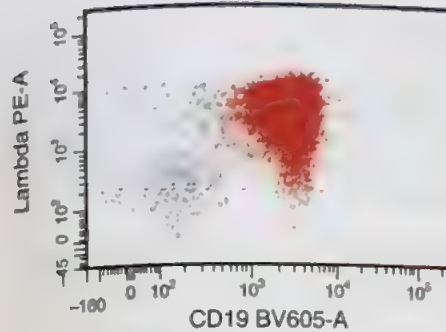
Mononuclear



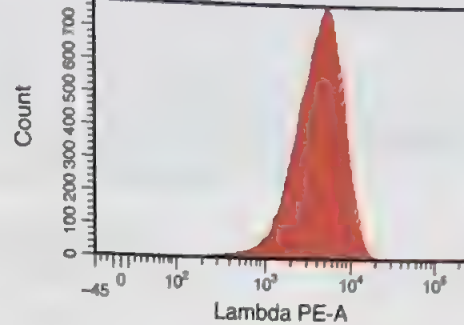
CD19+



Mononuclear

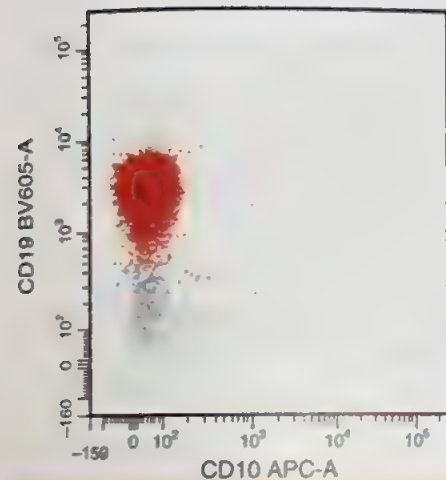


CD19+

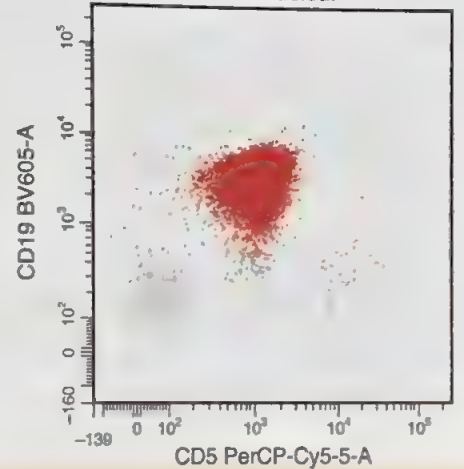


Population	#Events	%Total
Mononuclear	29,160	97.2
Lymphocytes	28,756	95.9
CD14+	7	0.0
CD19+	28,619	95.4

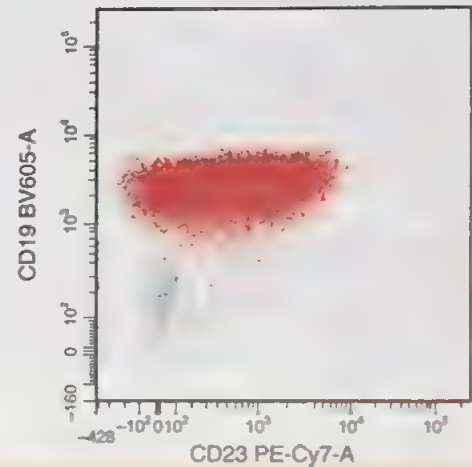
Mononuclear



Mononuclear



Mononuclear



QUESTIONS:

- What is the immunophenotype of this patient?
- What cell type is affected?
- What would a normal immunophenotype be?
- What is the patient's diagnosis?

ANSWERS:

- The immunophenotype is CD19+, CD23+, CD5+, and Lambda+.
- B-Lymphocytes, as their immunophenotype is CD19+.

- Normal B-Lymphocytes are not CD5+, which is a T-Cell marker. They also exhibit *both* Kappa and Lambda cell surface markers.
- The patient's diagnosis is CLL, supported by absolute lymphocytosis of more than 5.0×10^9 cells μL , a prominence of mature lymphocytes and smudge cells, as well as the immunophenotype of CD5+, CD23+, and CD19+ B-Lymphocytes with clonal Lambda expression.

REVIEW QUESTIONS

- Which of the four main tasks related to flow cytometric analysis often requires the expertise of a specialty physician?
 - Sample preparation
 - Cytometer operation
 - Data analysis
 - Results interpretation
- Antibodies binding the same antigen are classified in the same:
 - Isotype
 - ABO group
 - CD group
 - HLA group
- Forward light scatter is a result of:
 - Cellular size
 - Internal components
 - Dilution of sample
 - Intensity of laser light
- Which of the following is used to restrict the data analysis to one population?
 - Compensation
 - Linear amplification
 - Gating
 - Logarithmic amplification
- An FSC/SSC plot displays two populations, A and B. Population A is to the left of population B. Based on this information, which statement is true?
 - Cells of population B are more granular than those in A.
 - Cells of population A are larger than those in B.
 - Cells of population A are smaller than those in B.
 - Cells in population A are more granular than those in B.

See answers at the back of this book.

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Molecular Techniques in Hematopathology

Margaret L. Gulley, MD

CHAPTER OUTLINE

Structure of DNA and RNA	Sequence-Specific DNA Fragmentation by Restriction Endonucleases	Future Prospects of Molecular Assays
Applications of DNA Technology in Laboratory Medicine	Molecular Procedures	Summary Chart
Sample Sources for Molecular Procedures	Polymerase Chain Reaction (PCR)	Case Study 35-1
Nucleic Acid Extraction	Reverse Transcription Polymerase Chain Reaction (RT-PCR)	Review Questions
DNA Extraction From Cells or Tissue	In Situ Hybridization to Tissue	References
RNA Extraction	Immobilized on Glass Slides	
Nucleic Acid Quantification	Fluorescence In Situ Hybridization	
	DNA Sequencing	

LEARNING OBJECTIVES

At the end of this chapter, the learner should be able to:

- | | |
|---|---|
| <p>35-1 Explain the structure of DNA and RNA.</p> <p>35-2 Assess the types of pathological conditions for which DNA and RNA technologies are currently utilized as a diagnostic tool.</p> <p>35-3 Contrast specimen requirements for DNA with those for RNA analysis.</p> <p>35-4 Name the types of specimens from which DNA and RNA can be isolated.</p> <p>35-5 Distinguish extraction techniques used for DNA isolation from those used to isolate RNA.</p> | <p>35-6 Differentiate the two main analysis techniques utilized in nucleic acid quantification.</p> <p>35-7 Describe restriction endonucleases.</p> <p>35-8 Compare and contrast the purposes for utilizing PCR, RT-PCR, in situ hybridization, fluorescence in situ hybridization, and DNA sequencing.</p> <p>35-9 Given a particular clinical application, propose the appropriate molecular procedure.</p> <p>35-10 Evaluate the expected technological improvements for nucleic acid analysis.</p> |
|---|---|

Gene tests are increasingly adopted for clinical use when they add value beyond microscopy and flow cytometry or immunohistochemical laboratory tests. More accurate and complete classification of disease promotes more effective treatment and enhanced detection of low-level disease. Additionally, more precise measurement of disease burden can help reveal how effective the treatment is.

Deoxyribonucleic acid (DNA) is the inherited substance that encodes information needed for cell structure and function. This information is actionable through an intermediary substance called **ribonucleic acid (RNA)**. DNA and RNA are collectively called *nucleic acid*. Analysis of nucleic acid in patient specimens is the foundation for the field of laboratory medicine called *molecular pathology*. This discipline of laboratory medicine is crucial for diagnosis and monitoring of certain inherited, infectious, and malignant hematologic diseases.

The laboratory methods most commonly implemented in clinical settings are **polymerase chain reaction (PCR)**, **in situ hybridization**, and **DNA sequencing**. To understand

how each method works, this chapter begins with a review of the structure of DNA and RNA. It examines specimen sources for molecular procedures, describes nucleic acid extraction and quantification, explains sequence-specific DNA fragmentation by restriction endonucleases, and details related molecular procedures. The chapter ends with a discussion of the future prospects of molecular assays.

Structure of DNA and RNA

Human DNA is packaged into 46 chromosomes, each of which is a large molecule formed from two long strands of **nucleotides**. If all 46 chromosomes were aligned end to end, they would be 3 billion nucleotides long and would stretch for greater than 2 meters. Remarkably, all 46 of these long molecules are present within every cell nucleus in the body, tightly coiled around proteins to form chromatin. Within these strands is encoded the biochemical information necessary for life.

Nucleotides are the building blocks of DNA, and there are four types—adenine, guanine, thymine, or cytosine. Two

strands of these nucleotides wrap around each other to form a double helix. According to the rules of complementary strand pairing, an adenine in one strand can bond only with a thymine in the other strand, and guanine can bond only with cytosine (Fig. 35-1).

Within the nucleotide sequences of DNA are functional units called *genes*. Any given human cell expresses only a fraction of its approximately 25,000 different genes, depending on the cell type and stage of differentiation. Gene expression results in RNA **transcription**, producing "transcript" molecules that resemble the gene from which they were coded, except that RNA is single-stranded and its nucleotides contain a ribose instead of deoxyribose component. Transcripts serve as templates for protein **translation**.

Advances in our understanding of the genetic basis of disease led to the development of laboratory tests targeting DNA or RNA in patient samples. These tests rely on our ability to identify disease-related sequences of DNA or RNA by "nucleic acid probes." A **probe** is simply a single-stranded segment of nucleic acid whose nucleotide sequence is complementary to the target sequence, allowing the probe to bind to its target DNA or RNA through a process called **hybridization**. The probe may be labeled so that it is detectable in a tube (liquid phase) or on a glass slide (solid phase) to serve as a marker of the target sequence (Fig. 35-2).



FIGURE 35-1 DNA is composed of two strands of nucleotides that are bound to each other through hydrogen bonds (depicted as diagonal bridges). Four types of nucleotides are present: adenine (A), thymine (T), guanine (G), and cytosine (C). The two strands of DNA are said to be “complementary” to each other since an A on one strand can bond only to a T on the other strand, and a G can bond only to a C. The strands are oriented in opposite directions with respect to their backbone, so each strand has a 5' and a 3' end. In the laboratory, a probe can bind to DNA after the two strands are dissociated from one another by heating them to near-boiling temperature (95°C) or by treating them with an alkaline solution (high pH).

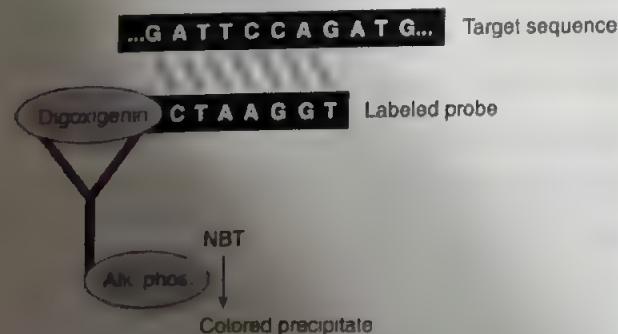


FIGURE 35.2 A probe represents a short strand of nucleotides that can bind (i.e., “hybridize”) to its complementary target nucleotide sequence. In the example depicted here, the probe was labeled with digoxigenin so that it could be subsequently detected via a colorimetric reaction using an antibody to digoxigenin.

CRITICAL THINKING QUESTION

35-1 Why is it helpful to leverage our understanding of genetics in laboratory analysis?

See answers to all Critical Thinking Questions at the back of this book.

Applications of DNA Technology in Laboratory Medicine

DNA technology is useful for diagnosing inherited diseases that, by definition, have a genetic basis and infectious diseases for which foreign DNA or RNA indicates that a pathogen is present. A list of pertinent heritable and infectious disease assays amenable to molecular diagnosis is shown in Box 35-1.

DNA technology also helps diagnose and classify various types of cancer because virtually all cancers harbor genetic defects responsible for malignant transformation. An altered gene that is activated to form cancer tissue is called an *oncogene*, or it is called a *tumor suppressor gene* if it is inactivated to form cancer. Laboratory detection of cancer-associated genetic defects not only contributes to improved diagnosis of affected patients so that the most appropriate treatment may be selected, but it also helps us monitor the efficacy of treatment by providing tumor markers to quantify in serial specimens.

Each of the previously mentioned clinical applications relies on prior basic and clinical research that has defined disease-specific genetic alterations and established molecular probe assays to detect those alterations. In the remainder of this chapter, we describe and compare common molecular technologies. Applications of these methods are found throughout this text.

BOX 35-1 Hematologic Diseases Amenable to Molecular Diagnosis

Inherited Diseases

- Hemoglobinopathies (sickle cell anemia, thalassemias)
- Hereditary hemochromatosis
- Bleeding disorders (factor VIII deficiency, hemophilia, von Willebrand's disease)
- Thrombotic disorders (factor V Leiden mutation, hyperhomocysteinemia, prothrombin mutation)
- Immunodeficiency states (DiGeorge or Wiskott-Aldrich syndromes)
- Red cell membrane disorders (hereditary spherocytosis)

Infectious Organisms

- Epstein-Barr virus (EBV)
- Cytomegalovirus (CMV)
- Human herpesvirus 8 (HHV8)
- Human immunodeficiency virus (HIV)
- Human T-lymphotropic virus type 1 (HTLV1)
- Malaria
- Parvovirus B19
- SARS-CoV-2 (Covid)

Sample Sources for Molecular Procedures

DNA is a very stable molecule. For example, fragments of DNA have been recovered from fossils and bodies that were mummified thousands of years ago. Nevertheless, specimens submitted to clinical laboratories for molecular genetic assays should be handled with care to preserve intact DNA. DNA is well preserved in fresh blood and marrow aspirates collected in ethylene diaminetetraacetic acid (EDTA) anticoagulant. For solid tissue biopsies, one of the best fixatives for preserving intact DNA is ethanol, though the more commonly used formalin fixative paraffin-embedded tissue block is also suitable unless the downstream test requires long strands of intact DNA.¹ Unlike DNA, RNA is unstable and easily degraded by ubiquitous natural enzymes collectively termed *RNases*, requiring more meticulous care to preserve target analytes.

Nucleic Acid Extraction

DNA or RNA is readily isolated from blood, marrow, body fluids, and solid tissue samples. To extract DNA from blood samples, nucleated blood cells are retained after lysing the more abundant red blood cells that lack nuclei and, hence, contain no chromosomes. In contrast to blood specimens, solid tissue specimens contain fewer red cells, so nucleated cell enrichment procedures are not required. Instead, tissues are minced, or they are sliced with an instrument called a *microtome* before nucleic acid extraction.

DNA Extraction From Cells or Tissue

DNA is separated from lipids and proteins by first treating with detergent and proteinase enzymes to lyse cell membranes and degrade proteins. The proteinase is subsequently heat-inactivated so that it does not interfere with subsequent enzymatic reactions. Commercial kits and automated instruments facilitate extraction of DNA by utilizing salt solutions to precipitate DNA, or else columns or glass/magnetic beads selectively bind DNA. The purified DNA is suspended in water and stored at 4°C short term or at $\approx -20^\circ\text{C}$ indefinitely.

RNA Extraction

Because *RNase* enzymes are ubiquitous, special precautions are required to prevent RNA degradation. In particular, gloves are changed frequently, and all solutions and plasticware must be *RNase*-free. Dedicated laboratory areas and reagents are recommended. Commercial preservative solutions are available to promote RNA stabilization and/or inhibit endogenous *RNase* activity. RNA is separated from cellular lipids and proteins by similar extraction techniques described earlier for isolating DNA. If not immediately proceeding to analysis, RNA resuspended in water is stored at $\approx -20^\circ\text{C}$ since cold temperature thwarts *RNase*.

CRITICAL THINKING QUESTION

35.2 Why is it important that specimens for nucleic acid testing not get too warm?

Nucleic Acid Quantification

DNA and RNA are quantified by spectrophotometry, which relies on the fact that nucleic acids absorb ultraviolet light of optical density (OD) 260 nm, whereas proteins absorb at OD₂₈₀. Alternatively, DNA is quantified using a fluorometer instrument that relies on emission of fluorescence by a dye when it binds DNA.

Sequence-Specific DNA Fragmentation by Restriction Endonucleases

A historic breakthrough in our ability to manipulate DNA was the discovery of naturally occurring enzymes that chop DNA into small fragments. Each enzyme cuts DNA only at specific nucleotide sequences, and this restricted ability to cut resulted in the name *restriction endonuclease*. These enzymes are normally produced by bacteria as a means of defending from foreign pathogens such as viruses. In clinical laboratories, we use these enzymes to reproducibly cleave DNA into smaller fragments to simplify manipulation of an otherwise excessively large molecule.

Examples of enzymes used for sequence-specific cleavage of DNA are *EcoRI*, derived from *Escherichia coli* and recognizing 5'-GAATTC-3'; *BamHI*, derived from *Bacillus amyloliquefaciens* and recognizing 5'-GGATCC-3'; and *HindIII*, derived from *Haemophilus influenzae* and recognizing 5'-AAGCTT-3'.

If a patient's DNA contains a **mutation** in the target sequence, then the number and/or size of the fragments is altered accordingly. In a clinical assay to detect such an alteration, a control cut site is evaluated in parallel with the disease-related mutation site to ensure that endonuclease digestion proceeded to completion.

Molecular Procedures

This section details the following molecular procedures: polymerase chain reaction (PCR), reverse transcription polymerase chain reaction (RT-PCR), in situ hybridization to tissue immobilized on glass slides, fluorescence in situ hybridization, and DNA sequencing.

Polymerase Chain Reaction (PCR)

PCR is the most common laboratory method for copying (i.e., amplifying) a short segment of DNA.² Target DNA can be copied a billion-fold so that it is more easily detected or further analyzed for disease-specific genetic alterations. PCR works by enzymatically replicating a particular segment of DNA amid all the DNA in a patient's sample. Segments as long as 40,000 base pairs (bp) have been amplified in research laboratories, but a more realistic limit in clinical samples is about 5,000 bp for intact DNA or about 500 bp if the DNA was extracted from fixed paraffin-embedded tissue.

Procedure

DNA is mixed with:

1. An enzyme called *DNA polymerase* that inserts nucleotides to convert single-stranded into double-stranded DNA,
2. Short probes (≈ 20 bases long) that flank the target sequence and serve as primers for initiation of DNA replication by the polymerase.

3. The building blocks needed for generating new DNA strands (dATP, dGTP, dCTP, dTTP), and
4. Buffer solution containing $MgCl_2$ that promotes polymerase activity.

The enzymatic reaction takes place in a thermocycler instrument programmed to vary the temperature of the reaction mixture sequentially so that progressive cycles of DNA replication can occur. After about 30 cycles, which takes only a couple of hours, approximately 1 billion copies of the target DNA have been generated and can be easily identified on gel electrophoresis (Fig. 35-3). Alternative ways to identify the reaction product include hybridization to an internal probe or sequencing the product to look for a disease-specific genetic alteration.

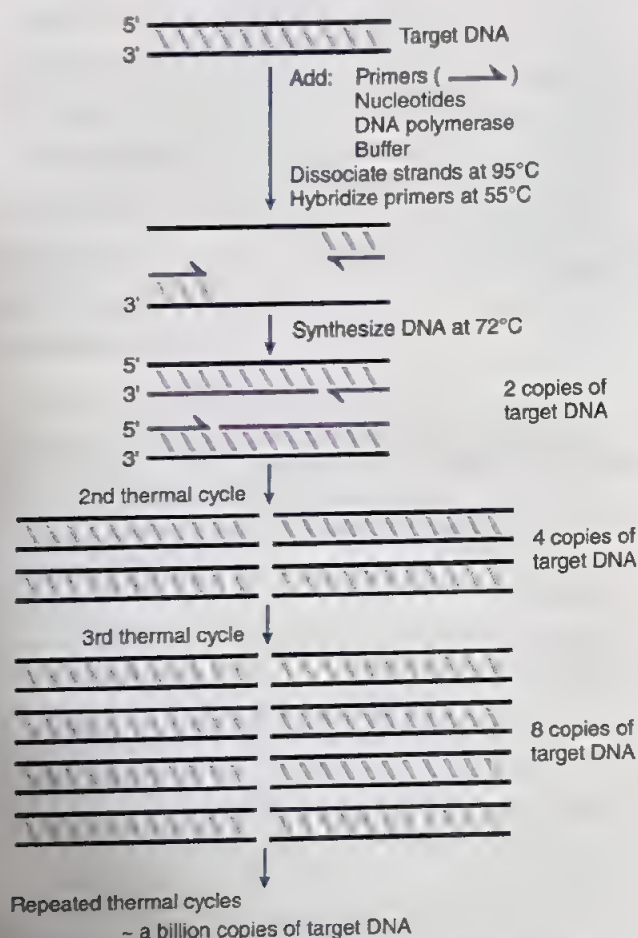


FIGURE 35-3 Polymerase chain reaction (PCR) is a method of copying a particular segment of DNA numerous times through a process of repeated cycles of heating, cooling, and DNA synthesis. To accomplish this, the target DNA is mixed with two short DNA probes called *primers* (shown as half-arrows) that are designed to span the segment of DNA to be amplified. Also added to the mixture is an enzyme called *DNA polymerase*, which converts single-stranded DNA into double-stranded DNA by incorporating nucleotides starting at the 3' end of each primer. A thermocycler instrument is programmed to heat and cool the sample sequentially. In each heat/cool cycle, the sample is first heated to 95°C to dissociate the two strands of DNA, and then cooled to 55°C to permit primer binding, then warmed to 72°C for enzymatic DNA replication. After the first cycle, an exact copy of the original target DNA has been produced. Then, in subsequent cycles, the products of previous cycles serve as templates for DNA replication, permitting an exponential accumulation of DNA copies (called *amplicons*). After 30 cycles, which takes only a couple of hours, a billion copies have been synthesized.

ADVANCED CONTENT

The DNA polymerase used in PCR reactions is "thermostable." This means that the polymerase can sustain near-boiling temperatures and still remain active. It is this feature that allows the reaction to continue through multiple heat cycles without loss of enzyme function. Thermostable polymerase is derived from bacteria such as *Thermus aquaticus* (Taq) that can survive in geysers.

Sample Requirements

Suitable sample types are solid tissue (fresh, frozen, fixed, or paraffin-embedded), blood, marrow, cytology fluid, plasma and other body fluids, and swabs. Minimum sample volumes are typically 1 mL of blood or body fluid, 0.5 mL of marrow, or 0.1 mm³ of tissue. Even fragmented DNA may be suitable because just one intact target sequence is required for the reaction to proceed.

Assay Time

The process takes approximately 1 day; further testing of the reaction product requires additional time.

Sensitivity

This has the highest sensitivity of available tests. Theoretically, one target is sufficient. In practice, amplification of one target per 10⁵ cells is achievable.

Specificity

Specificity is high if the assay is well designed.

Cost per Test

The cost per test is approximately \$100 if one sample plus controls are analyzed, less if samples are batched, and more if further testing of the PCR product is required.

Variations

Real-Time PCR In a variation of the PCR method called *real-time PCR*, a fluorescent internal probe is added to the reaction so that amplified products can be quantified at the end of every amplification cycle, and this information alongside results for reference materials can be used to extrapolate how much target DNA was present in the original sample³ (Fig. 35-4). In a second methodological variation called *multiplex PCR*, two or more different target DNAs are amplified in the same reaction. The second reaction may be a control amplification of an endogenous or spiked DNA that serves to verify that extraction and amplification worked as expected. A third variation called *melt-curve analysis* detects a mutation in amplified DNA based on evaluating the temperature at which an internal probe melts away from its complementary strand (Fig. 35-5).

Digital PCR In a variation of the PCR method called *digital PCR*, the preamplification mixture is divided into tens of thousands of tiny volumes in droplets or wells. Postamplification, the proportion of separate reactions with evidence of a PCR product is counted. This information is used to calculate the concentration of target DNA in the original sample.⁴

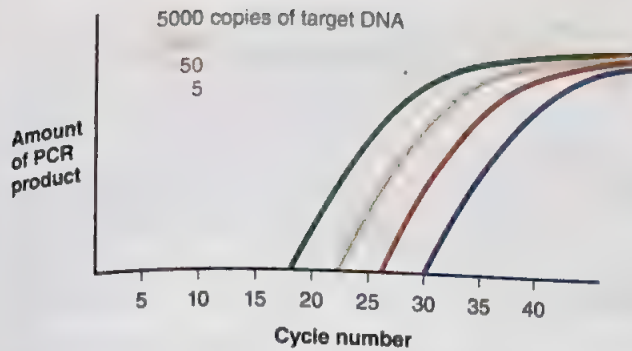


FIGURE 35-4 Real-time polymerase chain reaction quantifies target DNA in a patient sample. This is achieved in a thermocycler instrument that includes a detector to measure fluorescence at the end of each amplification cycle. When a fluorochrome-labeled internal probe in the mixture hybridizes to the PCR product, it generates a signal in proportion to the level of the product. In the amplification plot shown, the level of template in a given patient sample is estimated by extrapolation to a series of standards. Because the reaction vessels are never opened after amplification, the risk of amplicon contamination is minimized. Other benefits include low cost and fast turnaround time.

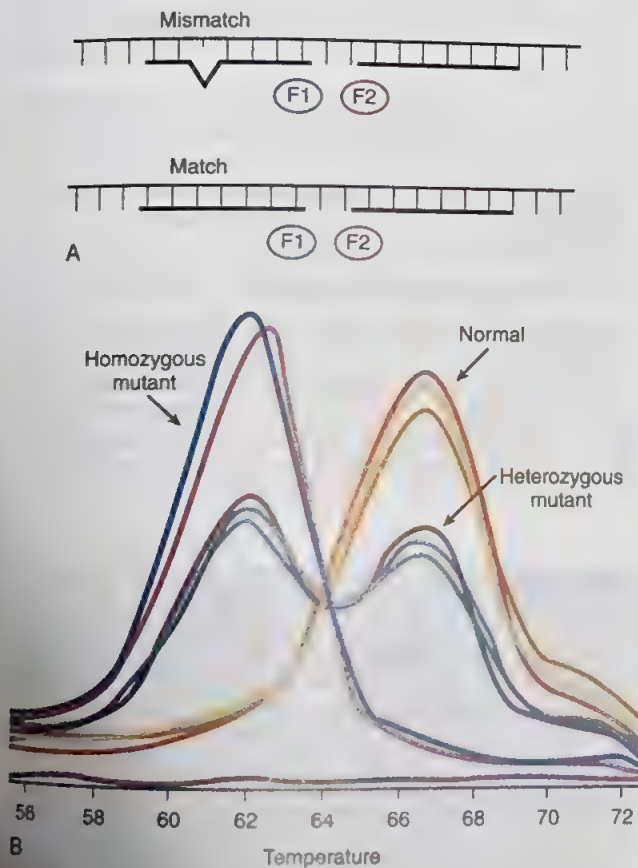


FIGURE 35-5 Melt curve analysis reveals a mutation in PCR-amplified DNA. A. Two fluorochrome-labeled, sequence-specific probes bind in tandem to a PCR product. Gradual heating results in probe dissociation which generates a measurable fluorescence signal. The temperature at which the probe dissociates depends on whether a mutation is present. B. The melt curve reflects changes in fluorescence as the temperature rises; multiple patient samples are classified as normal or mutant (heterozygous versus homozygous) for the *HFE* 845G>A mutation. The G to A nucleotide substitution at position 845 in the coding sequence causes Cys282Tyr amino acid substitution in the encoded protein to alter iron absorption from the diet.

Clinical Applications

1. Detect foreign nucleic acid characteristic of infectious disease.
2. Measure viral load in serial blood plasma specimens so that the efficacy of antiviral interventions can be monitored in infected patients.⁵
3. Selectively amplify DNA that has a particular tumor-associated gene defect, such as the *JAK2* mutation characteristic of myeloproliferative neoplasia,⁶ or the *IGH::BCL2* translocation characteristic of follicular and large cell lymphoma subtypes.
4. Measure tumor burden to monitor the efficacy of anticancer therapy, for example, to quantify *BCR::ABL1* transcript levels in serial blood collections from a patient with chronic myeloid leukemia. After treatment is complete, detect tumor markers to predict relapse of cancer before there is clinical or morphological evidence that minimal residual disease is present.⁷
5. Detect clonal immunoglobulin or T-cell receptor gene rearrangement characteristic of lymphoid leukemia or lymphoma, in contrast to the polyclonal gene rearrangements found in normal tissue or reactive lymphoid hyperplasia. (Fig. 35-6).⁸
6. Inherited disorders, such as sickle cell anemia in a prenatal specimen, can be tested for the causative mutation.⁹
7. Characterize DNA polymorphisms for forensic purposes or to help resolve misidentified samples in the clinical laboratory. Polymorphisms are natural differences in nucleotide sequences that distinguish DNA of one person from another. A "DNA fingerprinting" test reveals a pattern of polymorphisms that is unique to each person, except identical twins.

ADVANCED CONTENT

One cautionary note is that polymorphisms might interfere with clinical molecular tests for disease-associated genes. For example, a polymorphism at the site where a primer should hybridize could yield false negative PCR results for a pathogen genome. To overcome this pitfall, it is prudent to study the clinical performance of each laboratory test and to design additional confirmatory tests when indicated.

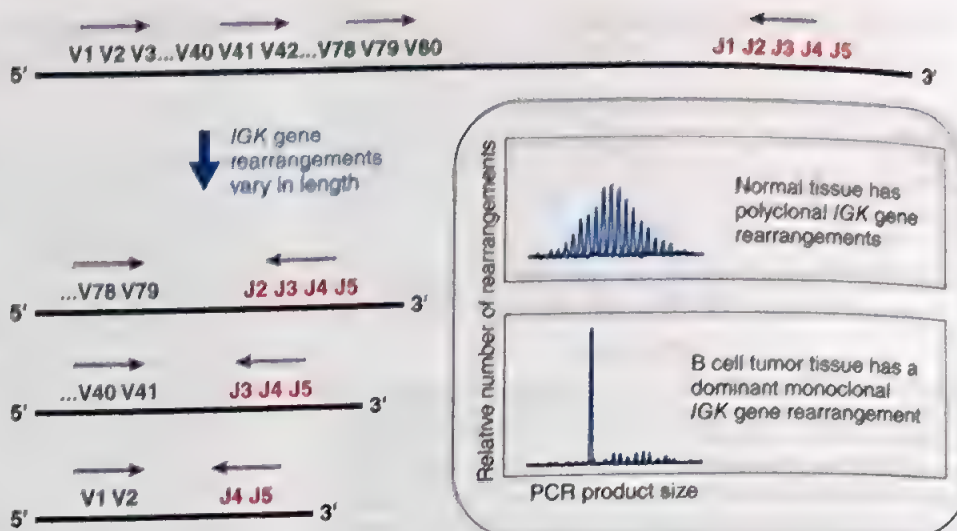
Major Advantages

- Extremely sensitive to low numbers of target sequences.
- Specific for the target gene of interest, particularly if a confirmatory method is added such as an internal probe or downstream sequencing of the PCR product.
- A broad range of specimen types is suitable.
- Small sample size requirement.
- Relatively fast and inexpensive unless extensive postamplification analysis is required.

Major Disadvantages

- Because the test is so sensitive to low levels of DNA template, extreme precautions are required to avoid contamination of samples or reagents by extraneous DNA.

FIGURE 35-6 Lymphocyte clonality determined by polymerase chain reaction (PCR). A. The immunoglobulin kappa light-chain gene (IGK) is rearranged in every B lymphocyte so that the cell can encode a unique antibody. Each B cell rearranges its IGK gene differently, and the size of the PCR product differs among normal B cells. In contrast, all of a patient's malignant B cells contain the same IGK rearrangement. PCR using primers spanning the rearranged gene segments helps distinguish B cell leukemia or lymphoma having **monoclonal** DNA from normal B cells having **polyclonal** DNA. In an analogous way, every T-cell tumor has monoclonal T-cell receptor gene rearrangement.



- Contamination by the abundant products generated from previous reactions is a worrisome problem.
- Meticulous care is required to maintain the integrity of each sample and reagent.¹⁰

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The purpose of reverse transcription polymerase chain reaction (RT-PCR) is to detect RNA in a patient specimen to evaluate (1) expression of a particular gene product, (2) RNA viruses, or (3) low-level disease as RNA can be more abundant than the DNA from which it was encoded.

Procedure

First, RNA is extracted from tissue and a complementary DNA (cDNA) is produced by the enzyme reverse transcriptase in the presence of primers and deoxyribonucleotides (Fig. 35-7). The primers show the enzyme where to initiate cDNA production. If these primers are composed of short random sequences, then the resultant cDNA represents all the RNAs in the sample, whereas a long sequence-specific primer targets the particular RNA of interest. After cDNA production, conventional PCR is carried out to amplify the cDNA segments of interest.

Sample Requirements

Suitable sample types and sizes are the same as for PCR. Because RNA is easily degraded, samples must be processed with care as described earlier, and controls are designed to check specimen quality.

Assay Time

The process takes approximately 1 day.

Sensitivity

Sensitivity is high. Theoretically, one target is sufficient.

Specificity

Specificity is high if the assay is well designed.

Cost per Test

The cost per test is approximately \$100 if only one sample is analyzed, less for batched samples.

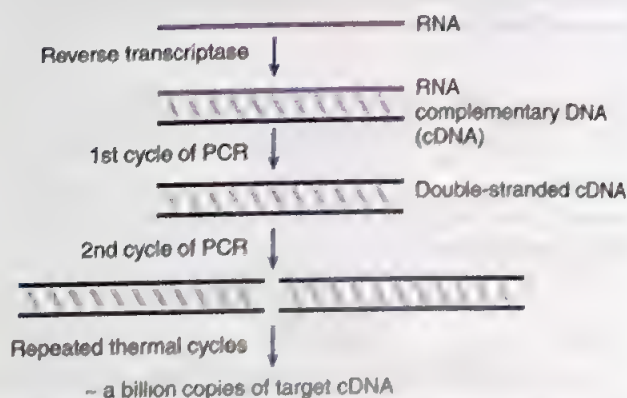


FIGURE 35-7 The reverse transcription polymerase chain reaction (RT-PCR) procedure is a method for detecting a particular RNA transcript. First, RNA serves as a template for construction of complementary DNA (cDNA) by the enzyme reverse transcriptase. Then routine PCR is done to amplify that sequence so that it may be measured or further analyzed. RT-PCR is a sensitive, specific, cost-effective, and rapid means of evaluating disease-associated RNA in a patient sample.

Clinical Applications

1. Detect a tumor-specific translocation that encodes chimeric RNA transcribed across the translocation breakpoint fusing two genes. Examples of fusion transcripts detectable by RT-PCR are t(15;17) *PML::RARA*; t(9;22) *BCR::ABL1*; t(8;21) *RUNX1::RUNX1T1*; t(2;5) *NPM1::ALK*; t(4;11) *KMT2A::AFF1*; and inv16 *CBFB::MYH11*.¹¹ Compared with PCR, RT-PCR is often more robust for detecting these translocations since breakpoint cluster regions are narrower after the natural process of RNA splicing removes introns. Furthermore, RT-PCR can detect translocations that are cryptic by karyotype.
2. Quantify minimal residual disease after cancer therapy using RT-PCR to amplify tumor-specific transcripts. This approach is used to monitor tumor burden and predict relapse of chronic myeloid leukemia, acute promyelocytic leukemia, and *NPM1* mutant acute myeloid leukemias.¹²

3. Detect a foreign organism using a probe targeting abundantly transcribed sequences, such as EBER RNA amply made by the Epstein-Barr virus DNA genome.¹³
4. Detect a virus having RNA genome, such as human immunodeficiency virus (HIV), human T-lymphotropic virus type 1 (HTLV1), hepatitis C virus (HCV), or SARS-CoV-2.¹⁴

Major Advantages

- Same advantages as PCR.

Major Disadvantages

- RNA is labile, so meticulous care is required to avoid degradation by RNases.
- Precautions are required to avoid contamination of samples or reagents by extraneous RNA, cDNA, or products from previous reactions. Physical separation of pre- and postamplification work areas is recommended.¹⁰

In Situ Hybridization to Tissue Immobilized on Glass Slides

The purpose of **in situ hybridization** is to localize DNA or RNA, as visualized by microscopy, in cells, or tissue immobilized on a glass slide.

Procedure

Fresh cells placed onto coated glass slides are fixed by immersion in alcohol, whereas paraffin sections on coated slides are immersed in xylene to diminish the wax. Next, protease treatment permeabilizes cell membranes, allowing the target DNA or RNA to become accessible for hybridization. The slides are incubated for several hours in a mixture of labeled probes (complementary to the sequence of interest) at a temperature that optimizes hybridization. Unbound probe is washed away, and bound probe is then localized using a signal detection process. Counterstaining permits microscopic visualization of the target nucleic acid in the context of histologic features and cytological detail (Fig. 35-8).¹⁵

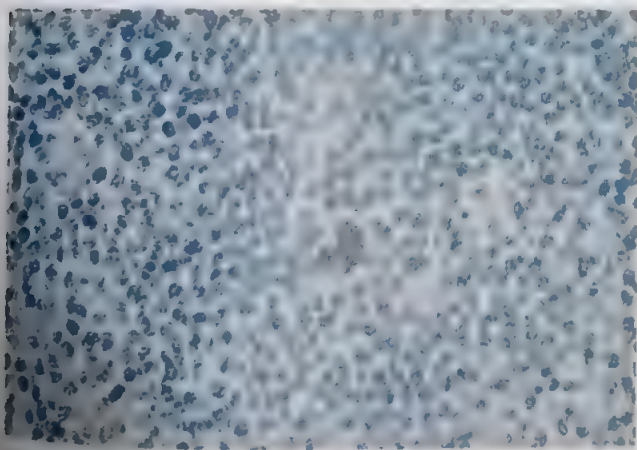


FIGURE 35-8 The *in situ* hybridization technique permits visualization of nucleic acid in tissue sections on glass slides. In this example, hybridization to Epstein-Barr virus encoded RNA (EBER) transcripts reveals that the viral gene product is localized to the nucleus of a Reed-Sternberg cell in a case of Hodgkin lymphoma. In contrast, the background lymphocytes stain only with a green counterstain.

Sample Requirements

Samples consist of glass slides with cells isolated from a body fluid or sections from a block of formalin-fixed paraffin-embedded tissue. Decalcified tissue is generally not suitable if acid was used to soften bony material.

Assay Time

The process takes approximately 2 days.

Sensitivity

Sensitivity is moderate. Theoretically, one target is sufficient, but in practice the signal-to-noise ratio is improved when there are multiple targets.

Specificity

Specificity is high.

Cost per Test

The cost per test is approximately \$200.

Clinical Applications

1. Localize foreign organisms in relation to lesional cells. This is especially important for pathogens that might also exist as "normal flora," such as HHV8, human papillomavirus, or Epstein-Barr virus.¹⁶
2. Detect and localize gene expression by targeting messenger RNA transcripts. For example, hybridization to kappa and lambda immunoglobulin light chain RNAs generates a cleaner histologic signal for lymphoid clonality than does immunohistochemistry for light chain proteins since those proteins are secreted from cells.¹⁷

Major Advantages

- Permits visualization of target nucleic acid in the context of cytologic and histologic features.
- Sensitive to low numbers of affected cells in the sample (even a single cell).

Major Disadvantages

- The assay is more robust when the target analyte is abundant (e.g., when there are multiple copies of Epstein-Barr virus or human papillomavirus genomes per infected cell).¹⁸

Fluorescence In Situ Hybridization

The purpose of **metaphase fluorescence in situ hybridization (FISH)** is to detect and localize specific DNA sequences in **metaphase chromosomes** using a probe labeled in such a way that it fluoresces in response to an input light. In dual hybridizations, one probe might fluoresce green while another probe fluoresces red, thus allowing both gene targets to be localized for definitive identification of a chromosomal translocation or deletion.

Procedure

Metaphase spreads of chromosomes are prepared on glass slides using the same methods that are employed in karyotyping. The two chromosomal DNA strands are denatured (e.g., separated from each other) using heat or a strong base and then allowed to hybridize to a labeled probe complementary to the sequence of interest. Unbound probe is washed away, and bound probe marking the sequences of

interest is visualized as a bright dot by microscopy. Counterstaining permits simultaneous visualization of chromosome morphology to further interpret medical significance of the result.

Sample Requirements

Fresh cells that are capable of mitosis (e.g., cell division).

Assay Time

The process takes approximately 1 to 2 days.

Sensitivity

Sensitivity is high (one target is sufficient).

Specificity

Specificity is high (long probes bind tightly and exclusively to their complementary DNA target).

Cost per Test

The cost per test is approximately \$400.

Interphase FISH Variation

Interphase FISH is done on intact cell nuclei or on a paraffin-embedded tissue section immobilized on a glass slide. Protease treatment removes protein so that long probes can more readily diffuse to bind their complementary target sequence inside the nucleus. The slide is visualized by microscopy so that the number and pattern of signals in each nucleus can be interpreted in the context of histomorphology.

In healthy cells, there are two copies of each gene (one inherited from the mother, the other from the father), whereas fewer or more gene copy numbers characterize an inherited disease having germline variants that the patient was born with or that characterize a tumor having somatic variants acquired in neoplastic cells that proliferate uncontrolled. Interphase FISH is used to count gene copy number that confers prognosis in cancers like myeloma or chronic lymphocytic leukemia. Interphase FISH performed with two probes on either side of a structural break in DNA can confirm translocation partners such as *IGH::MYC* in Burkitt or in large cell lymphoma and to further test for coexisting *IGH::BCL2* or *IGH::BCL6* implying diagnosis of an aggressive *double-hit lymphoma*.¹⁹ In suspected chronic myeloid leukemia, FISH is the single best test to evaluate if *BCR::ABL1* translocation is present.²⁰

Clinical Applications

1. FISH uses fluorescent probes to detect DNA alterations and specific RNA targets used for genetic counseling, diagnosis, and treatment options.

Major Advantages

- FISH permits visualization of target DNA in the context of karyotypic visualization of metaphase chromosomes to help identify structural alterations that are difficult to define by karyotype alone. As an example, DiGeorge syndrome is characterized by a tiny deletion within chromosome 22 that is detectable by FISH but is difficult to visualize by karyotype (Fig. 35-9).

Major Disadvantages

- Metaphase analysis requires live cells, so fresh specimens must be promptly transported to the laboratory.

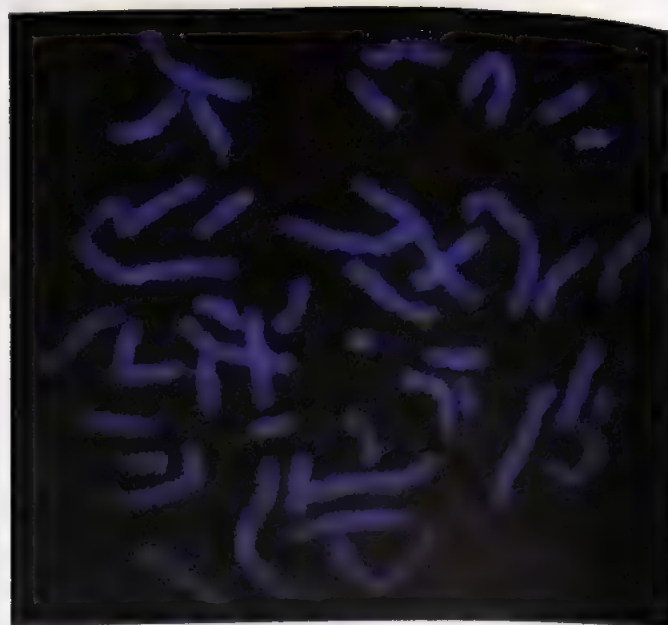


FIGURE 35-9 Fluorescence in situ hybridization (FISH) supports diagnosis of DiGeorge syndrome, a congenital immunodeficiency caused by partial deletion of chromosome 22. In the example shown here, bright fluorescent signals identify the two number 22 chromosomes from among the 46 chromosomes in a cell. The DiGeorge probe and a control probe are visible on the normal chromosome 22 (lower left); however, only the control probe is seen on the other chromosome 22 (upper right) consistent with a deletion of target DNA. (Photomicrograph courtesy of Kathleen Kaiser-Rogers, PhD, University of North Carolina.)

- Visualization of fluorochromes requires a microscope with a specialized light source, and the signal fades with time.

DNA Sequencing

DNA sequencing is incredibly powerful for revealing medically relevant variants across large swaths of genomes. Generating the nucleotide sequence of dozens to millions of selected gene regions in a patient specimen yields a catalog of variants, including single nucleotide variants (SNVs, also called point mutations), insertions or deletions (also called indels or copy number variants), and structural variants (e.g., translocations). By comparing a patient's variants to sequences found in healthy and diseased cohorts, the medical significance of each variant is interpreted.

Procedure

In **massive parallel sequencing** (also called **NextGen sequencing** or **NGS**), multiple segments of DNA are pulled out of the DNA specimen to enrich for gene regions of interest by PCR-amplification with or without first capturing target DNA fragments using probes. Additional sequences are incorporated on both ends of the PCR amplicons to (1) immobilize amplicons on a surface within a sequencer instrument and (2) distinguish one patient from another and optionally one natural molecule from another so that batch sequencing of many different molecules from different patients is feasible in a single sequencing run. Within the sequencing instrument, each specimen-specific "library" of amplicons is then copied, and a detector tallies which nucleotide was incorporated next as DNA polymerase proceeds to sequentially add

nucleotides. The same library of amplicons is copied again and again during additional sequencing cycles, generating millions of “reads” that are aligned to the reference genome to identify their gene region. Differences from the reference sequence are cataloged in a “variant call file.” After filtering out noise and population polymorphisms, the clinical significance of the remaining variants is interpreted in light of the input specimen, quality metrics such as “depth of coverage” at a given **locus**, known disease associations, and the clinical status of the patient.^{23,24}

Sample Requirements

Suitable sample types and sizes are the same as for PCR.

Assay Time

The process takes approximately 5 days.

Sensitivity

Sensitivity is high. In practice, detection of one in 100 cells is typically achievable.

Specificity

Specificity is highest among all tests.

Cost per Test

The cost per test is approximately \$2,000 if one sample plus controls are analyzed, less if samples are batched.

Variations

RNAseq Variation Sequencing RNA helps classify disease and predict its behavior in response to therapy by revealing expression profiles of multiple RNA transcripts. In this procedure, extracted RNA from a patient specimen is reverse transcribed to make cDNA, which in turn is sequenced using the same procedure described earlier for DNA sequencing. Results show the extent to which each gene is overexpressed or underexpressed. The pattern of expression across thousands of genes is matched to a database of known patterns for purposes of classifying the disease. For example, in diffuse large B cell lymphoma, RNA expression profiles help assign the cell of origin, which has prognostic utility.

Also identified by RNAseq are structural changes in RNA that predict response to therapy, such as RNA splice aberrations predicting efficacy of splicing inhibitor drugs in myelodysplastic syndrome or *PML-RARA* fusion transcripts predicting response to retinoic acid inhibitors in acute promyelocytic leukemia.

Compared with RT-PCR or in situ hybridization, RNAseq evaluates thousands of analytes at once to bolster confidence in disease classification. Furthermore, studying aberrant biochemical pathways allows us to better understand disease pathogenesis and selection of therapy to thwart relevant biochemical pathways.

Sanger Sequencing Variation Sanger sequencing was the gold standard molecular method for many decades before massive parallel (NextGen) sequencing proved to be more sensitive to low-level variants and more broadly informative of patient status. Nevertheless, Sanger sequencing is still used to resolve questionable results of other gene tests. It is also a cost-effective method of sequencing when only one or two

variants require testing. For example, once the gene variants of a patient with beta thalassemia are identified by massive parallel sequencing, other family members can be tested for the particular familial mutations by Sanger sequencing.

In the Sanger procedure, PCR enriches a segment of the genome, then DNA polymerase copies those amplicons starting at a bound primer. The size of the resulting copy is linked to whether A, T, G, or C was just incorporated. Gel electrophoresis separates the copies by size to reveal their nucleotide sequence.

Clinical Applications

1. Detect mutations of the *HBB* (beta-globin) gene to help diagnose beta-thalassemia, to predict disease severity, and to detect thalassemia carriers at increased risk for having an affected child. Online information about this and other tests for heritable disorders is found in The Genetic Testing Registry at <https://www.ncbi.nlm.nih.gov/gtr>.
2. Study familial predisposition to hematologic malignancy.²⁵
3. Sequence the commonly mutated genes associated with myeloid neoplasia to assist in diagnosis, prognosis, predicting response to therapy, and monitoring affected patients.²⁶
4. Sequence the kinase domain of *ABL1* DNA to predict drug responsiveness or resistance in patients with chronic myeloid leukemia treated with kinase inhibitors.²⁷
5. Perform HIV **genotyping** to predict which drugs will be effective based on the spectrum of mutations present in the viral genome.²⁸

Major Advantages

- In many diseases, mutation sites vary among affected patients, and DNA sequencing can evaluate all relevant genomic regions in a single assay.
- Sequencing is considered the gold standard assay for characterizing heritable or somatic variants in the human genome.
- Identify pathogens across all species and genera (e.g., the microbiome) to predict outcome of hematopoietic cell transplantation.²⁹

Major Disadvantages

- Expensive with a longer turn-around time and greater requirement for professional expertise than simpler or more automated gene technologies.³⁰

CRITICAL THINKING QUESTION

- 35-3** What is often the greatest preanalytical and analytical concern with nucleic acid analysis?

Future Prospects of Molecular Assays

Molecular technology is a powerful tool in clinical laboratories. In the coming years, we expect technological improvements to yield faster and less-expensive methods of nucleic acid analysis. At the same time, new discoveries related to

the genetic basis of human disease are fostering development of even more tests for a wider range of disease states. Moreover, tailored treatment is now chosen based on pharmacogenetic tests that assess patient DNA to predict which

drug is most likely to be effective or least likely to cause adverse side effects. It seems likely that this technology will revolutionize laboratory diagnosis and management of many diseases.

SUMMARY CHART

- Deoxyribonucleic acid (DNA) is a double-stranded molecule composed of long sequences of nucleotides. In the laboratory, the two strands of DNA can be denatured into single-stranded components.
- A DNA probe is a segment of single-stranded DNA that is complementary to the target sequence of interest.
- All nucleated cells of the body contain the same set of genes, although any given cell expresses only a small fraction of those genes.
- Genetic variants found in inherited diseases and cancer can be detected by a molecular assay.
- Each species of infectious organism shares unique genomic sequences, and molecular tests can detect the genomes of foreign pathogens in human tissues.
- DNA or RNA is the substrate for molecular tests. DNA and RNA can be extracted from any tissue or body fluid.
- Polymerase chain reaction (PCR) is a method of DNA amplification that is capable of producing 1 billion copies of a particular segment of double-stranded DNA, rendering exquisite sensitivity to detect low-levels biomarkers.
- Real-time polymerase chain reaction can quantify a particular DNA target to serve as surrogate for the extent of tumor burden or viral load. Thus, the efficacy of therapy can be monitored in serial specimens from a patient by measuring a disease-specific genetic marker.
- By combining PCR with melt-curve analysis in a real-time PCR instrument, rapid detection of a sequence variant is feasible.
- RNA can be converted to cDNA, then amplified by PCR and further analyzed.
- In situ hybridization is a means of localizing target DNA or RNA to a particular cell or tissue structure visualized by microscopy.
- Fluorescence in situ hybridization uses labeled probes to visualize a particular segment of DNA within whole chromosomes.
- DNA sequencing is considered the gold standard assay for defining genetic variants.

CASE STUDY 35-1

A 45-year-old male had liver cirrhosis, diabetes, high serum ferritin, and high transferrin saturation. He was diagnosed with hemochromatosis, a disease characterized by high iron levels that can damage organs and cause cirrhosis and diabetes. To evaluate the underlying cause of his disease, a blood test was performed for mutation of the *HFE* gene responsible, at least in part, for hereditary hemochromatosis. In his results shown in Figure 35-5, PCR and melt curve analysis revealed a mutation (*HFE* 845G>A) that was homozygous, meaning it was present in both alleles of the *HFE* gene (i.e., both maternal and paternal inheritance). The mutant *HFE* gene encodes abnormal HFE protein (amino acid substitution Cys282Tyr) predisposing him to iron overload via overabsorption of iron from his diet. He was treated with therapeutic phlebotomy (meaning intentional blood loss) until his serum iron levels returned to the normal range. He spoke with a genetic counselor and learned that other family members may be at risk for iron overload if they, too, inherited mutant *HFE* genes.

QUESTIONS

1. What was the benefit of adding molecular testing to this patient's clinical evaluation?
2. How might family members use this information?

ANSWERS

1. Without the analysis for the *HFE* gene, the physician would not know conclusively the underlying cause for the patient's hemochromatosis. Additionally, the results provide an opportunity for useful genetic counseling for the patient and their family members.
2. Blood relatives of an affected family member can be counseled about the risks and benefits of testing for disease-related familial mutation(s). PCR with melt curve analysis is a cost-effective choice to detect a point mutation such as the *HFE* 845G>A mutation. DNA findings can be predictive of future disease so that disease prevention strategies can be considered.

REVIEW QUESTIONS

- All assays using nucleic acid probes rely on which of the following principles?
 - DNA is different in every cell of a particular individual.
 - A probe hybridizes to its complementary sequence.
 - Heat or high pH converts single-stranded to double-stranded DNA.
 - Probes bind to RNA rather than DNA.
- Which of the following procedures is used to convert double-stranded DNA into two single strands?
 - Heat DNA to 95°C
 - An acid pH solution
 - DNA polymerase
 - Reverse transcriptase
- Which of the following steps is part of a massive parallel sequencing procedure?
 - Color precipitation
 - Immobilize tissue sections
 - Library preparation
 - Spread metaphase chromosomes
- Which of the following enzymes can copy DNA?
 - EcoRI*
 - DNA polymerase
 - Ethanol
 - Formalin fixative
- Which of the following substances is most likely to become degraded in a patient sample stored at room temperature overnight?
 - RNA
 - Lipid
 - Amino acid
 - DNA
- Which of the following probes would be most appropriate for an assay in which you want to target the following DNA sequence, 5'-AAAGGGTCTCTCTTTGGG-3'?
 - 5'-AAACCCCTCTCTCAAAGGG-3'
 - 5'-TTTCCCAGAGAGAAAACCC-3'
 - 5'-CCCAAAGAGAGACCCTTT-3'
 - 5'-GGGAAACTCTCTCCCCAAA-3'
- Which statement accurately describes the polymerase chain reaction (PCR)?
 - It is a method for amplifying a particular segment of DNA.
 - The function of a primer is to cut DNA into many fragments.
 - It is labor-intensive and expensive compared with sequencing.
 - Turn-around time is rapid since no probes are required.
- In addition to patient samples, every PCR run includes a control sample containing all of the reagents necessary for DNA amplification except for target DNA. How is this "no template" control used?
 - To ensure that no reagent was left out of the reaction
 - To ensure that there are no inhibitors of DNA amplification
 - To show that PCR products can be generated
 - To check for contamination by extraneous DNA
- Which reagent is most critical for designing a real-time PCR assay that measures *BCR::ABL1* as opposed to *PML::RARA* translocation?
 - Primers
 - DNA polymerase
 - Magnesium chloride
 - Deoxyribonucleotides
- Which concept best distinguishes polymerase chain reaction (PCR) from reverse transcription PCR (RT-PCR)?
 - The forward *versus* reverse direction of strand synthesis
 - DNA polymerase *versus* RNA polymerase catalyzes the reaction
 - Generate a DNA product *versus* an RNA product
 - Patient DNA *versus* RNA is the substrate for the reaction
- Which of the following statements is accurate concerning the in situ hybridization procedure for analyzing paraffin tissue sections immobilized on glass slides?
 - DNA or RNA is localized in the context of histologic and cytologic features.
 - Restriction fragment length polymorphisms are amplified.
 - Since the human genome is in every cell, only microbes are usefully targeted
 - Formalin fixed tissue interferes with hybridization, but frozen tissue is suitable
- In situ hybridization is most effectively used to do which of the following steps?
 - Localize beta-globin protein in a tissue section
 - Find clonal rearrangement of the *IGH* gene
 - Localize Epstein-Barr virus to malignant cells
 - Resolve which patient is the source for a misidentified specimen
- Which assay is most sensitive for detecting minimal residual disease in a patient treated for chronic myeloid leukemia?
 - Karyotype
 - Reverse transcription polymerase chain reaction
 - Fluorescence in situ hybridization
 - Light microscopy

Continued

REVIEW QUESTIONS—cont'd

14. Molecular probes can be used to speciate infectious agents in patient samples primarily because:
 - a. Probes targeting pathogens never cross-react with human genomic DNA.
 - b. Drug resistance factors differ in each species.
 - c. Each species shares unique sequences that differ from those of other species.
 - d. Dividing organisms often acquire genetic mutations.
15. Which assay is most appropriate for routine clinical detection of the mutation responsible for sickle cell anemia?
 - a. In situ hybridization
 - b. Fluorescence in situ hybridization
 - c. Gene expression profiles by RNAseq
 - d. Polymerase chain reaction with melt-curve analysis
16. Which of the following specimen types is most robust for DNA extraction?
 - a. EDTA anticoagulated blood sample
 - b. Residual protein remaining after hemoglobin electrophoresis
 - c. Serum collected via clot activator tube
 - d. Whole blood collected without anticoagulant
17. In the future, nucleic acid analysis is predicted to:
 - a. Slow innovation of novel technologies
 - b. Become more expensive
 - c. Become more focused on tailored pharmacogenetic treatments
 - d. Become restricted to a smaller range of diseases

See answers at the back of this book.

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Answers to Chapter Critical Thinking Questions

Chapter 1: Morphology and Maturation of Human Blood Cells: Hematopoiesis

- 1-1 Neutrophils are a type of white blood cell that is important for combating bacterial infections. An increase in the production and release of neutrophils, also known as neutrophilia, is a common response to bacterial infections. Additionally, an increase in band neutrophils, which are immature forms of neutrophils, may be observed. This is often referred to as a "left shift" in the white blood cell count and can indicate an acute infection.
- 1-2 Reduction of nuclear size; condensation of chromatin; change in nuclear shape; appearance and disappearance of primary granules; appearance of secondary granules; color changes in cytoplasm; and change in overall size of cell.
- 1-3 CSFs play a crucial role in supporting and enhancing the body's immune response, particularly in situations where the immune system is compromised due to cancer, immunodeficiency syndromes, or anemia. These patients lack the production of certain blood cell lines. Introduction of CSFs can stimulate cellular proliferation so these patients can replace affected cell populations.
- 1-4 Determining cell surface markers provide valuable insights into the composition of cell populations and aids in the understanding and management of various disease states.

Chapter 2: The Red Blood Cell: Structure and Function

- 2-1 Glycophorin is responsible for maintaining the red blood cell's net negative charge and is often a site of antigen attachment. Spectrin is critical for red blood cell membrane integrity and its deformability.
- 2-2 Control of ion permeability is linked to the regulation of cell volume. Maintaining a stable cell volume is essential for the proper function of red blood cells. Dysregulation of ion permeability can lead to swelling or shrinkage of the cells, affecting their ability to transport oxygen and carbon dioxide efficiently.
- 2-3 The cells can compensate by shifting the oxygen dissociation curve to the right, making RBCs more efficient in oxygen delivery.
- 2-4 RBCs are anucleated, limiting their metabolic processes. They do not contain a mitochondrial apparatus. Additionally, the main function of the RBC is to deliver oxygen, not to consume it.

Chapter 3: Bone Marrow Structure and Function

- 3-1 No, an increased neutrophil concentration alone would not typically indicate an immediate need for a bone marrow study. Neutrophilia, or an elevated neutrophil count, can be a normal response to various conditions, such as infections, inflammation, stress, or certain medications. In many cases, neutrophilia is a part of the body's natural immune response.
- 3-2 Bone marrow needs to be evaluated and slides need to be made immediately while the marrow sample is fresh and unclotted.
- 3-3 The significance of the Prussian blue stain for bone marrow samples lies in its ability to identify and characterize iron stores within cells, specifically in the form of ferric iron or hemosiderin. The Prussian blue stain is highly sensitive to iron, and it specifically stains ferric iron deposits blue, providing valuable information about iron stores in the bone marrow.

Chapter 4: Examination of the Peripheral Smear: Red Cell, White Cell, and Platelet Morphology

- 4-1 The number of CBC samples that require manual blood smear review is 10% to 25%, and the accurate review of blood smears remains a crucial diagnostic aid for patients.
- 4-2 It is vital that oil immersion is utilized to identify key cellular characteristics such as nuclear chromatin pattern that is only seen clearly under oil immersion 100 \times magnification. The 40 \times scan allows for a broader evaluation of more cells, which allows the morphologist to evaluate for distribution of findings across many or only a few cells for adequate grading.
- 4-3 Ensure that the blood smear is properly prepared. Rouleaux formation is typically reversible when the sample is diluted. Adding saline or another diluent can disperse rouleaux, indicating that it's an artifactual phenomenon. True agglutination is usually seen as clumps of cells under the microscope. Consider the patient's clinical condition and repeat the testing with a fresh sample.
- 4-4 Anemia can cause red blood cells (RBCs) to be hypochromic due to a decrease in the concentration of hemoglobin within the cells. Hemoglobin is responsible for giving RBCs the pink-red color; therefore, a low amount of hemoglobin will result in lighter colored cells upon staining, which may be indicated by a larger than normal central pallor.
- 4-5 Schistocytes are fragmented red blood cells that can be observed on a peripheral blood smear, and their presence is often associated with hemolytic processes. The term "schistocyte" is derived from the Greek word "schistos," meaning split or cleft. These abnormal RBC fragments can be a result of mechanical damage to red blood cells within the circulation.
- 4-6 If a clotted blood sample is submitted for a complete blood count (CBC), it is more likely to result in a falsely decreased platelet count rather than an elevated one. The process of clotting can lead to platelet activation and consumption within the clot, resulting in a lower platelet count in the liquid portion of the sample. On a peripheral blood smear prepared from a clotted sample, you might observe platelet clumping, where platelets are aggregated together.

Chapter 5: Quality Management in the Hematology Laboratory

- 5-1 You could share with your friend that every laboratory has a systematic plan for quality. There are measurements taken throughout each day to provide confidence in patient results. No new method, analysis, or instrument is implemented without quality experiments. Additionally, laboratories are regulated by governing bodies that provide metrics with which all testing and policies must uphold. All of these elements work together to provide assurance to physicians, other health-care professionals, and the public that results are of the highest quality.
- 5-2 Interference experiments introduce realistic interference substances into the testing environment (e.g., lipemia and hemolysis) at varying concentrations to determine concentrations of validity when they are present in patient samples. With replication experiments, we can repeat samples until we demonstrate reliable precision over short- and long-term runs by observing the same or a very close result over and over.
- 5-3 A delta check signifies a significant change in a patient's results from the current run to the last reported run. This can indicate an

interfering substance like lipemia or hemolysis, an instrument issue, or a true change in the patient's status. When evaluated, delta checks provide a rich internal quality assurance indicator.

Chapter 6: Anemia: Diagnosis and Clinical Considerations

- 6-1 The reference ranges for each laboratory depend on the institution or medical center and the population of patients they serve.
- 6-2 The RBCs, white blood cells, and platelets; a pancytopenia.
- 6-3 The reticulocyte count would be decreased in iron deficiency anemia and megaloblastic anemia as a result of ineffective erythropoiesis.
- 6-4 Hemoglobin, hematocrit, RBC indices, RBC count, reticulocyte count, and examination of the peripheral smear.
- 6-5 Because anemia is treated according to its cause(s), the cause should be considered and determined before beginning either supportive therapy (such as a transfusion) or replacement therapy. Transfusions can obscure and confuse the findings of diagnostic tests in patients with anemia. It is important that a diagnosis be made, if at all possible, before transfusions are given.

Chapter 7: Iron Metabolism and Hypochromic Anemias

- 7-1 Of course, as patients begin to respond to therapy their RBC, HGB, and HCT values will begin to increase, and RBC indices will start to normalize. Increased reticulocyte numbers, seen as polychromasia on the peripheral blood smear and reticulocyte corpuscular hemoglobin (CHr), are early indicators of a positive erythropoiesis response. The vast majority of iron is absorbed in the duodenum and first portion of jejunum. If either are removed, an individual could lack appropriate absorption sites. This could lead to developing iron deficiency anemia and other nutritional deficiencies.
- 7-2 The body's regulation mechanisms for iron trigger the liver to increase production of transferrin when iron serum levels begin to decline, resulting in more binding sites available.
- 7-3 Inflammatory conditions may cause leukocytosis. Specifics of a particular inflammatory condition may influence the white blood cells in a number of different ways.
- 7-4 Basophilic stippling is a classic finding with lead toxicity, which is a cause of sideroblastic anemia. Lead prevents the breakdown of RNA in reticulocytes, and leads to the grouping of undegraded ribosomes, causing basophilic stippling. Basophilic stippling can be seen in a variety of anemias, but it is most prevalent when associated with sideroblastic anemia, due to the effects of lead poisoning.

Chapter 8: Megaloblastic Anemias and Other Macrocytic Anemias

- 8-1 Megaloblastic anemia is defined as an anemia due to impaired DNA synthesis. Because all the different types of blood cells derive from nucleated pluripotent hematopoietic stem cells, it follows that any impairment of DNA synthesis will affect all blood cell types (as well as other cells of the body), resulting in decreased numbers of WBC, RBC, and platelets. Additionally, there is the destruction of cells in the marrow due to dyspoiesis. Yes, because the patient has significantly lower red blood cells, they present with fatigue, pallor, and weakness due to the anemic state.
- 8-2 Folic acid has a higher turnover in the body and a higher rate of loss compared with vitamin B₁₂. More demographic groups are at risk of not getting enough folic acid, including people living in poverty, pregnant women, lactating women, elderly adults, people with alcoholism, and individuals with chronic diseases. In addition, individuals who overcook their food may eliminate the folic acid, as it is not resistant to heat. Additionally, vitamin B₁₂ storage rate is high, and it takes several years for a person to develop vitamin B₁₂ deficiency as a result of decreased intake and malabsorption.
- 8-3 Pernicious anemia is most often caused by malabsorption, while folate deficiency is most often caused by nutritional deficiency.

Knowing the difference can aid diagnosis because peripheral findings for both are often similar, making the critical analysis of patient history important. In addition, treatment choice and duration will vary significantly depending on the cause, and the anemia may be a comorbidity or secondary to another disease process (myelodysplastic, leukemia, alcoholism, and genetic mutation, to name a few).

Chapter 9: Hemolytic Anemias: Intracorporeal Defects: Hereditary Defects of the Red Cell Membrane

- 9-1 The diagnosis of hemolytic anemia involves a systematic approach that includes clinical evaluation, laboratory tests, and sometimes additional diagnostic procedures. Figure 9-2 outlines one flow chart and suggested steps to follow. The cause of hemolysis should be determined. A Coombs' test can determine an immunological cause. Nonimmunological causes consist of morphological abnormalities found on examination of the peripheral smear. Hemolytic anemias often show features such as schistocytes (fragmented cells), spherocytes, and polychromasia. A medical history, including symptoms such as fatigue, pallor, jaundice, dark urine, and an enlarged spleen should be taken, and a physical examination performed. Then family history and pertinent confirmatory tests will help make a definitive diagnosis.
- 9-2 Genetic mutations alter either the amount or function of cellular proteins. Alterations change the integrity of the red blood cell membrane and alter the cell's morphology. Typically affected proteins include spectrin, anion exchanger 1 (AE1), Ankyrin, and Protein 4.1R.
- 9-3 Jaundice, anemia and splenomegaly. Jaundice would be caused by increased levels of bilirubin circulating in the patient. Anemia is caused by the increased destruction of red blood cells. The condition is characterized by the presence of spherocytes which are less deformable than normal disc-shaped RBCs. An enlarged spleen would be caused by the organ's increased load sequestering frail red blood cells from circulation because of their abnormal membranes.

Chapter 10: Hemolytic Anemias: Intracorporeal Defects: Hereditary Enzyme Deficiencies

- 10-1 G6PD deficiency causes premature destruction of cells through intravascular, acute hemolysis. Fragmented cell structures such as bite- or helmet-shaped cells may be present in peripheral blood, indicating early destruction of red blood cells.
- 10-2 PK deficiency causes extravascular hemolysis, in which the spleen removes affected cells. This is evidenced by anemia, rapid increases in reticulocyte counts, nucleated red blood cells, and polychromatic red blood cells, all indicating prolonged peripheral circulation of deficient cells.
- 10-3 The enzyme activity is only decreased in hereditary conditions.

Chapter 11: Hemolytic Anemias: Intracorporeal Defects: The Hemoglobinopathies

- 11-1 Many of the unstable hemoglobins have high oxygen affinity and, therefore, may not cause anemia, making diagnosis in this group of patients particularly difficult.
- 11-2 In sickle cell trait, the heterozygous form of the disease, individuals inherit both a normal β -globin gene and a sickle globin gene (β^s). As a result, individuals with sickle cell trait produce both normal HbA and HbS, with a predominance of HbA in an approximate ratio of 60:40. Additionally, cells that are homozygous for hemoglobin s sickle when the oxygen level is decreased to 4% to 6% whereas cells heterozygous for hemoglobin s (sickle cell trait) won't sickle until the oxygen level is decreased to 2%.
- 11-3 Electrophoresis is part of a comprehensive approach to the diagnosis of hemoglobinopathies, often used alongside other techniques such as high-performance liquid chromatography

(HPLC) and molecular testing. Combining multiple methods enhances the accuracy and reliability of the diagnosis. Electrophoresis not only identifies the presence of abnormal hemoglobins but also allows for the quantification of different hemoglobin fractions. This is essential for assessing the relative amounts of normal and abnormal hemoglobins.

Chapter 12: Hemolytic Anemias: Intracorporeal Defects: **Thalassemia**

12-1 The hemoglobinopathies are a result of a qualitative change in globin chains caused by a change in the amino acid sequence of the globin chain. The amount of globin chains is normal, but the globin chains do not function normally. The thalassemias are a result of a quantitative change in globin chains. The globin chains are structurally normal, but the amount and ratios of globin chains are abnormal.

12-2 While a definitive diagnosis of Thalassemia often requires additional laboratory tests, such as hemoglobin electrophoresis or DNA analysis, etc., a trained lab scientist examining a peripheral blood smear can observe certain morphological features that may raise suspicion of thalassemia. These include: microcytic hypochromic RBCs, target cells, anisopoikilocytosis, nucleated RBCs, and basophilic stippling (not specific to thalassemia). It's important to note that while peripheral blood smear analysis can provide valuable information, it is not sufficient for a definitive diagnosis of thalassemia. Confirmatory tests are typically required to identify the specific hemoglobin variants associated with thalassemia.

12-3 Because the beta thalassemias are the result of genetic mutations, and almost 400 different beta thalassemia mutations exist, different combinations of mutations seen in patients lead to a spectrum of different clinical presentations. To simplify the disorders, we categorize them into beta thalassemias major, intermedia, and minor.

12-4 The thalassemias classically present with decreased MCV, MCH, and possible decreased MCHC, with a normal RDW. The MCV is disproportionately low compared with the hemoglobin and hematocrit values. The RBC count is increased relative to the hemoglobin value which is due to the presence of a higher number of smaller red blood cells, compensating for the decreased hemoglobin content per cell.

12-5 Thalassemia minor and iron deficiency anemia may initially present with similar hematologic findings, including decreased RBC indices, microcytic, hypochromic red blood cells, ovalocytes, and target cells. However, it is essential to differentiate the two disorders using iron studies and possibly hemoglobin electrophoresis in order to avoid unnecessary treatment of iron supplementation. A patient who is inappropriately given iron supplementation is at risk of iron-overload and subsequent complications.

Chapter 13: Rare Normocytic Normochromic Anemias: **Aplastic Anemia and Related Disorders and Paroxysmal Nocturnal Hemoglobinuria**

13-1 Aplastic anemia is a disorder characterized by a reduction in the number of hematopoietic stem cells in the bone marrow, leading to a decrease in the production of all blood cell types, including red blood cells, white blood cells, and platelets. The reticulocyte count, which reflects the rate of red blood cell production, is typically low in aplastic anemia because it cannot produce RBCs as a normal BM can.

13-2 Aplastic anemia is characterized by a failure of the bone marrow to produce an adequate number of blood cells, whereas PNH is characterized by complement-mediated hemolysis of red blood cells due to a genetic mutation affecting the cell membrane.

Chapter 14: Hemolytic Anemias: Extracorporeal Defects

14-1 Increased levels of bilirubin and lactate dehydrogenase (LD), along with a positive direct antiglobulin test (DAT), are

indicative of hemolytic anemia caused by an immune process. Bilirubin is a by-product of hemoglobin catabolism, which occurs when red blood cells lyse and hemoglobin is released. Lactate dehydrogenase (LD) is an enzyme found in various tissues, including red blood cells. LD increases because it is released from the red cell during hemolysis.

14-2 ABO blood group antibodies include IgM class antibodies which trigger intravascular hemolysis due to complement, where other blood group antibodies are more often IgG, leading to sensitization of red blood cells and extravascular hemolysis.

14-3 Spherocytes in the peripheral blood smear is a strong indicator of extravascular hemolysis. These cells have lost their biconcave shape and are now shaped like a sphere. This can happen when there has been damage to the red blood cell membrane such as seen when the spleen has attempted to pluck it out of circulation due to sensitization.

Chapter 15: Anemia Associated With Systemic Diseases

15-1 You would expect the ESR to be increased in anemia of chronic kidney disease because of increased inflammation.

15-2 No. The decreased number of reticulocytes observed in hypoproliferative anemias corresponds with a decreased amount of polychromasia observed on a blood smear.

Chapter 16: Benign White Blood Cell Disorders

16-1 The increased epinephrine will allow the marginating pool of neutrophils to merge into the circulatory pool. Therefore, increasing the circulating number of WBCs counted for the CBC.

16-2 Yes. IM is transmitted orally, so any shared mouth device can transmit the virus.

16-3 Yes. Reactive lymphocytes, also known as atypical lymphocytes can be observed on a peripheral blood smear of a patient infected with cytomegalovirus (CMV).

Chapter 17: Introduction to Leukemia and the Acute Leukemias

17-1 Acute leukemia tends to have a sudden onset and can have a rapidly fatal clinical course if left untreated. It can present with pancytopenia or with marked leukocytosis with circulating blasts. Chronic leukemia tends to have an insidious onset and indolent clinical course. Many times, it is found incidentally when evaluation for unexplained or persistent leukocytosis. In acute leukemia the cells present are immature cells with a marked increase in blasts (>20% blasts). Chronic leukemia is characterized by markedly increased number of mature forms.

17-2 Patients with acute leukemia tend to present with anemia and thrombocytopenia. The white blood cell count can be decreased or markedly increased with circulating blasts present.

17-3 Although the FAB classification required 30% blasts for the diagnosis of AML, the newer WHO classification requires 20% blasts. The WHO classification is now standard for diagnosis of AML.

17-4 A bone marrow biopsy should be done. The aspirate obtained during the bone marrow procedure should be sent for flow cytometry analysis to help differentiate between acute myeloid leukemia and acute lymphoblastic leukemia. Cytochemical stains can also be helpful in differentiating between myeloid and lymphoid origin. FISH, cytogenetics studies, and molecular studies should be obtained to help classify acute leukemia based on cytogenetic abnormalities and to evaluate for mutations with potential treatment implications.

17-5 Yes, AML with t(8;21), AML with inv(16) or t(16;16), and acute promyelocytic leukemia with t(15;17) are considered acute leukemia regardless of the blast percentage.

Chapter 18: Myeloproliferative Neoplasms I: Chronic Myelogenous Leukemia

18-1 The stem cells responsible for myeloid cell production include the Myeloid stem cell, which gives rise to the CFU-GEMM (colony forming unit – granulocyte, erythrocyte, megakaryocyte, and monocyte) stem cell. The CFU-GEMM supports the production of the CFU-G (colony forming unit-granulocyte), the CFU-M (colony forming unit-monocyte), the CFU-EO (colony forming unit-eosinophil), and the CFU-BASO (colony forming unit-basophil). The myeloid stem cell and CFU-GEMM are multipotential stem cells and specific cell lineage lines are targeted by the more targeted unipotent stem cells.

18-2 Anemia identification is a result of prolonged hypoxia in the tissues. General clinical symptoms include pallor, malaise, and weakness. These symptoms are a result of the decreased amount of oxygen delivered to the tissues either due to qualitative or quantitative issues of hemoglobin and/or erythrocyte production.

18-3 Anemia is typically identified by the patient's low RBC count, hemoglobin, and hematocrit values. Normocytic anemia also takes into consideration the MCV value—normocytic anemia is identified by the patient having a MCV value within normal range.

Chapter 19: Myeloproliferative Neoplasms II: Polycythemia Vera, Essential Thrombocythemia, and Primary Myelofibrosis

19-1 In PV, there is an increase in the total number of red blood cells, white blood cells, and platelets. However, the proportion of plasma (the liquid component of blood) is not increased at the same rate. As a result, the blood becomes more viscous (thicker), which can impede the sedimentation of red blood cells. The elevated viscosity may hinder the normal settling of red blood cells in an ESR test, leading to a lower-than-expected ESR.

19-2 The hematology analyzer will likely erroneously identify giant platelets as leukocytes or raise an error flag triggered by microclots, reflecting to more manual procedures for peripheral cell review.

19-3 Primary myelofibrosis (PMF) is a chronic myeloproliferative neoplasm characterized by the proliferation of abnormal bone marrow cells, fibrosis (scarring) of the bone marrow, and the potential for transformation to acute leukemia. Because the fibrosis in the bone marrow forces the body to find sites outside the bone marrow to produce cells, this leads to extramedullary hematopoiesis. The first site of extramedullary hematopoiesis is the spleen.

Chapter 20: Myelodysplastic Syndromes

20-1 Sideroblasts contain iron, which isn't obvious on the regular Wright stain utilized for bone marrow aspirate evaluation. Prussian blue staining is required to visualize iron more definitively.

20-2 When the bone marrow is hypocellular, analysis for cell morphology and identification can be more difficult due to there not being enough cells to visualize or the aspirate being difficult to obtain during the aspirate procedure.

Chapter 21: Chronic Lymphocytic Leukemia and Related Lymphoproliferative Disorders

21-1 In CLL, the abnormal B cells are often mature but functionally impaired. The B cells in CLL do not contribute to humoral immunity as they should and thus cause a lack of immunoglobulins that negatively affects immunity making patients with CLL more susceptible to bacterial and viral infections.

21-2 CLL: small, mature lymphocytes and smudge cells present | Hairy cell: fried-egg appearance to lymphocytes | reactive lymphocytosis: lymphocytes with abundant cytoplasm | ALL: lymphoblasts present | SCCL: irregular-shaped nucleoli with irregular defects, notches, and folds in nucleus | Adult T cell

leukemia/lymphoma: cloverleaf nuclear shape in lymphocytes | plasma cells dyscrasia: plasma cells present.

Chapter 22: The Lymphomas

22-1 Similar cells can be seen in a variety of benign and malignant conditions other than Hodgkin lymphoma.

22-2 All chromosomal abnormalities, deletions, mutations, translocations, or amplifications are damaged DNA leading to unregulated cell proliferation.

22-3 Not necessarily; indolent NHLs may be slow growing, but are often incurable, whereas aggressive NHLs can have rapid fatality rates if left untreated. HL has a generally better prognosis compared to many types of NHL. Treatment protocols for HL are often highly effective, with high cure rates, especially in cases diagnosed at an early stage.

Chapter 23: Multiple Myeloma and Related Plasma Cell Disorders

23-1 The analysis of immunoglobulins, specifically IgM and IgG antibodies, can be valuable in distinguishing between initial infection and reinfection with a virus or foreign body. IgM antibodies are typically the first antibodies produced in response to a new infection. IgG antibodies are produced later in the immune response and provide long-term immunity. As the infection progresses and resolves, IgG levels increase and can persist in the bloodstream for an extended period. During reinfection, the presence of high levels of IgG without a significant increase in IgM suggests a memory response, indicating previous exposure to the antigen.

23-2 An M-spike indicates the presence of a monoclonal gammopathy, meaning that a single clone of plasma cells is producing a specific type of immunoglobulin (monoclonal protein). Further analysis is needed to identify the type of immunoglobulin and determine if it is associated with an underlying condition including monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma, or other plasma cell disorders.

23-3 The most common symptom upon presentation in most multiple myeloma patients is extreme bone pain. This is due to uncontrolled proliferation of plasma cells and increased osteolytic lesions, resulting in bone pain, pathological fractures, spinal cord compression, and hypercalcemia.

23-4 In Waldenström macroglobulinemia (WM), the increased viscosity of samples, especially when left at room temperature, is primarily due to the high levels of monoclonal immunoglobulin M (IgM) antibodies produced by the malignant lymphoplasmacytic cells. This monoclonal IgM is often present in elevated concentrations in the blood, contributing to the hyperviscosity syndrome associated with WM. Samples often have to be warmed up to 37°C before analysis.

Chapter 24: Lipid (Lysosomal) Storage Diseases and Histiocytosis

24-1 Gaucher's disease is a lysosomal storage disorder caused by a deficiency of the enzyme glucocerebrosidase, leading to the accumulation of lipid-laden macrophages (Gaucher cells) in the bone marrow. The infiltration of Gaucher cells can disrupt normal hematopoiesis, affecting the production of blood cells with the anemia developing gradually over time, and compensatory mechanisms may prevent the release of immature RBCs.

24-2 The large granules observed in leukocytes in these diseases represent accumulated lipids that cannot be properly processed due to the enzyme deficiencies. These granules can be visualized in peripheral blood smears and are indicative of the underlying lysosomal storage disorders (Mucopolysaccharidoses [MPS]). The large granules observed in leukocytes of patients with MPS are a result of the accumulation of undegraded sulfate material, which includes mucopolysaccharides, in the lysosomes of cells.

Chapter 25: Hemostasis

25-1 In von Willebrand's disease, the absence or dysfunction of vWF impairs the normal adhesion of platelets to the damaged blood vessel walls and the stabilization of factor VIII. This leads to difficulties in forming a stable platelet plug at the site of injury, resulting in prolonged bleeding. In Glanzmann's thrombasthenia, the absence or dysfunction of GP IIb/IIIa impairs the ability of platelets to aggregate and form a stable clot. This results in prolonged bleeding time, easy bruising, and a tendency for spontaneous mucocutaneous bleeding.

25-2 Hemostatic testing is typically ordered based on clinical indication. It is not necessary to perform a comprehensive hemostatic analysis on every patient unless there are specific symptoms, medical history, or clinical situations that warrant such testing. Clinical practice guidelines often provide recommendations on the appropriate use of hemostatic testing based on clinical scenarios, symptoms, and risk factors.

Chapter 26: Disorders of Primary Hemostasis: Quantitative and Qualitative Platelet Disorders and Vascular Disorders

26-1 Without enough platelets (150 to $400 \times 10^9/L$), an adequate platelet plug cannot form, resulting in continued bleeding from vascular injury and inadequate vascular healing.

26-2 The clinical course of thrombocytosis can vary widely among individuals, and the presence or absence of symptoms may not correlate directly with the platelet count. The risk of complications, particularly thrombotic events, is influenced by factors such as age, overall health, and the presence of additional risk factors.

26-3 Yes, patients can have the correct amount of platelets, but those platelets present are defective, resulting in platelets that are unable to activate, adhere, or aggregate appropriately to form the required platelet plug to complete primary hemostasis.

Chapter 27: Disorders of Secondary Hemostasis: Plasma Clotting Factors

27-1 The PT and PTT analysis is sensitive to the lack of enough fibrinogen preventing the formation of a clot. This results in more time needed for a clot to form during the analysis, or even no clot ever forming if fibrinogen levels are very low. The mixing study replaces the low or nonexistent fibrinogen levels with normal plasma, thus resulting in a "corrected" result.

27-2 Although the PT and PTT analyses don't pick up on vWF directly, the PTT is affected by changes in FVIII. Deficiencies in vWF affect FVIII, as they are strong cofactors and form a complex to promote successful clotting.

27-3 Inhibitors are antibodies. Because a person with Hemophilia A or B lacks Factors VIII or IX, respectively, when they are transfused these factors for therapy, the factors are likely to be recognized as foreign and produce an immunological response forming alloantibodies.

Chapter 28: Disseminated Intravascular Coagulation and Primary Fibrinolysis

28-1 The fibrinolytic system involves multiple inhibitors and components to ensure a delicate balance between coagulation (clot formation) and fibrinolysis (clot dissolution). Multiple inhibitors and components are involved to prevent excessive fibrinolysis, maintain hemostatic balance, and ensure appropriate responses to vascular injury and tissue repair.

28-2 DIC involves both bleeding issues and thrombotic issues. Platelet counts will be low and PT/aPTT results will be elevated, indicating bleeding problems, but the patient will also have thrombosis throughout the body.

28-3 If the fibrin deposition does not completely occlude the lumen of the damaged blood vessel, red cells may undergo a shearing effect as they traverse this area, with resultant fragmentation of the red blood cells. Microangiopathic hemolytic anemia with schistocytes may be evident on the blood smear in approximately 50% of patients with acute DIC.

28-4 The main goal of treatment is to treat or remove the underlying pathological stimulus and maintain blood volume and hemostatic function.

Chapter 29: Introduction to Thrombosis and Anticoagulant Therapy

29-1 Liver Disease can disrupt the delicate balance between clot formation and dissolution, leading to coagulopathy. Many factors and inhibitors are produced by the liver, so if the liver is unable to produce these substances, coagulation and fibrinolysis can be affected.

29-2 For females, this is often the time that many start to use oral contraceptives, which then increases their risk of thrombosis should they have an inherited condition.

29-3 For a patient with an acquired Factor VIII (FVIII) inhibitor, it's important to evaluate the presence of lupus anticoagulant, as these conditions can coexist. Mixing studies, which would remain uncorrected, as well as a prolonged aPTT due to LA if present.

Chapter 30: Body Fluid Examination: Analysis of Serous, Cerebrospinal, and Synovial Fluids

30-1 Slides must be prepared regardless of whether any cells are seen in the hemacytometer, as the potential to detect abnormal or malignant cells increases with the cytocentrifugation process.

30-2 Approximately 2–4 mL of fluid is collected into each of three to five sequentially numbered, sterile, nonadditive tubes. The tubes must be filled in numerical order. Tube #1 is used for chemical analysis, because it typically would be contaminated with peripheral blood and cellular debris from the initiation of the puncture, and it requires centrifugation before analysis. Tube #2 is used for microbiological analysis, as it is less affected by contamination from initiation of the LP. Tube #3 is sent to hematology for the cell count and differential analysis, as it is less affected by any bleeding from initiation of the LP. Additional tubes (#4, #5) may be used for further biochemical analyses, serology, cytology, flow cytometry, immunocytochemistry, or molecular genetic analysis.

30-3 Automated cell counters should not be used for CSF, because the allowable background limits of the analyzer diluent may be higher than the normal range for CSF cell counts.

Chapter 31: Hematology: Methods

31-1 Results in a hemolyzed sample tend to exhibit reduced red blood cell count, hemoglobin, hematocrit, and mean corpuscular volume (MCV). An increased red cell distribution width (RDW), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet count are often present.

31-2 Disposable plastic counting chambers are only used once, so it minimizes risk of specimen contamination due to inappropriate cleaning techniques. They are also designed to ensure uniformity of depth and specimen volume which can be erroneous on traditional counting chambers if loaded incorrectly or cover slip is placed on chamber incorrectly.

31-3 Yes, if the WBC count is abnormally high or low, there may be a great dissonance between the relative and absolute counts. The relative count represents the percentage of each type of leukocyte relative to the total white blood cell count and is expressed as a percentage. The absolute count provides the actual number of each type of leukocyte per unit volume of blood. In these circumstances, the absolute counts become a better representation of the actual differential picture.

Chapter 32: Principles of Automated Differential Analysis

32-1 RBC, HGB, HCT, MCV, mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

32-2 Forward angle light-scatter measures white blood cell size.

32-3 At least two levels of control material should be analyzed every 8 hours during each day of patient testing.

Chapter 33: Coagulation Methods

33-1 Hemolysis due to the release of ADP, which can activate platelets. Lipemia because the plasma could obscure the true optical density of the sample. Glass tubes should be avoided because platelets will activate and adhere to glass surface. It is important that the temperature remain at 37°C while testing and the pH between 6.5 and 8.5. Platelet-rich plasma should sit for half an hour before testing for platelets to regain responsiveness. Testing should occur within 3 hours of collection. And samples should be stirred so platelets can come together close enough to aggregate.

33-2 First, the patient would have an abnormal PT and/or aPTT result without a known cause for the abnormality. If only one is abnormal, specific factors could be deduced as deficient, making the next steps a bit more focused. The physician could then choose to run a mixing study and assess if the patient's PT and/or aPTT would correct when added to normal pooled plasma. A correction would indicate a factor deficiency. If there was not a correction, an inhibitor may be present. After the mixing study, specific factor or inhibitor assays could be ordered to assess for either a deficiency of a factor or presence of an inhibitor, allowing the physician to make a diagnosis.

33-3 An increased D-dimer, the detection of FDPs, prolonged PT and aPTT, decreased platelet count, and low fibrinogen together would all indicate DIC.

Chapter 34: Applications of Flow Cytometry to Hematopathology

34-1 During hydrodynamic focusing, the sample cell suspension is guided through the flow cell by sheath fluid in a way to produce a single cell stream through the flow cell interrogation point. This ensures that characteristics are recorded of one cell at a time. If the cell suspension is very concentrated, a high flow rate could cause multiple cells to pass the laser stream and produce false data.

34-2 If the new lot is >20% brighter than the old lot, the stain intensity can be lowered by way of titration.

Chapter 35: Molecular Techniques in Hematopathology

35-1 There are many disease states and infectious agents that laboratory analyses such as hematology findings and/or enzyme/substrate quantification are most useful as markers for inclusion in diagnostic decisions. Genetic testing allows for more definitive diagnostic data. It enables a shift toward precision medicine, where interventions are tailored to individual genetic profiles, leading to improved diagnosis, treatment, and overall patient care.

35-2 Nucleic acids are composed of proteins that can degrade when exposed to increased temperatures.

35-3 Contamination of the specimen with external DNA, RNA, or products from previous reactions is a significant concern in nucleic acid testing.

Answers to Review Questions Chs 1-35

CHAPTER 1

Q#	Ans	Objective
1	A	1-13
2	B	1-8
3	A	1-10
4	B	1-9
5	C	1-9
6	D	1-10
7	B	1-4
8	C	1-6
9	B	1-5
10	B	1-1
11	D	1-1
12	A	1-2
13	B	1-3
14	A	1-7
15	D	1-11

CHAPTER 2

Q#	Ans	Objective
1	C	2-10
2	C	2-1
3	A	2-4
4	B	2-5
5	D	2-5
6	B	2-4
7	D	2-7
8	C	2-11
9	A	2-12, 2-13
10	B	2-12
11	B	2-2
12	C	2-3
13	D	2-6
14	A	2-7, 2-8

CHAPTER 3

Q#	Ans	Objective
1	A	3-2
2	B	3-7
3	A	3-12
4	A	3-3
5	B	3-9
6	C	3-5
7	C	3-6
8	C	3-4
9	B	3-8
10	C	3-6
11	D	3-10
12	B	3-11
13	C	3-1

CHAPTER 4

Q#	Ans	Objective
1	A	4-1, 4-2
2	A	4-3
3	C	4-4
4	D	4-11
5	C	4-8
6	D	4-14
7	B	4-10
8	A	4-10
9	D	4-10
10	A	4-5
11	A	4-12
12	B	4-11
13	C	4-8
14	B	4-14
15	B	4-11
16	C	4-11
17	C	4-11

Continued

CHAPTER 4—cont'd

Q#	Ans	Objective
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19	D	4-14
20	B	4-14
21	C	4-3
22	C	4-9
23	A	4-6
24	H	4-9
25	L	4-7
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31	F	4-9
32	B	4-6
33	M	4-10
34	J	4-9
35	N	4-9
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Q#	Ans	Objective
1	B	5-1
2	C	5-3
3	D	5-2
4	A	5-5
5	A	5-5
6	B	5-12
7	C	5-7
8	A	5-14
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10	B	5-15
11	A	5-4
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13	D	5-4
14	B	5-6
15	C	5-1
16	A	5-6
17	A	5-8
18	B	5-12
19	A	5-10
20	B	5-15

CHAPTER 6

Q#	Ans	Objective
1	D	6-9
2	D	6-1
3	A	6-1
4	D	6-8
5	C	6-5
6	C	6-4
7	D	6-2
8	A	6-7
9	C	6-9
10	A	6-3
11	A	6-10

CHAPTER 7

Q#	Ans	Objective
1	D	7-1
2	B	7-2
3	C	7-2
4	A	7-4
5	C	7-3
6	B	7-7
7	B	7-4
8	A	7-7
9	B	7-4
10	C	7-5
11	D	7-6
12	A	7-8
13	C	7-9

CHAPTER 8

Q#	Ans	Objective
1	B	8-1, 8-9
2	A	8-6
3	B	8-2
4	D	8-4
5	B	8-5
6	C	8-3
7	D	8-6
8	D	8-7
9	C	8-8
10	B	8-12
11	D	8-8
12	A	8-10
13	B	8-11
14	B	8-12

CHAPTER 9

Q#	Ans	Objective
1	B	9-1
2	C	9-2
3	C	9-3
4	D	9-4
5	B	9-5
6	B	9-6
7	C	9-7
8	B	9-8
9	D	9-9
10	A	9-10
11	B	9-11

CHAPTER 10

Q#	Ans	Objective
1	B	10-1
2	A	10-2
3	D	10-3
4	D	10-4
5	A	10-5
6	C	10-6
7	C	10-7
8	B	10-3
9	D	10-9
10	C	10-8
11	C	10-2, 10-9
12	A	10-10

CHAPTER 11

Q#	Ans	Objective
1	C	11-1
2	B	11-2
3	B	11-3
4	C	11-4
5	A	11-5
6	B	11-6
7	B	11-7
8	D	11-8
9	A	11-9
10	C	11-10
11	B	11-11
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13	A	11-13
14	A	11-14
15	A	11-13
16	C	11-14
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CHAPTER 12		
Q#	Ans	Objective
1	C	12-2
2	D	12-3, 12-4
3	A	12-5
4	C	12-5
5	A	12-4
6	B	12-6
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9	B	12-7, 12-8
10	B	12-8
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CHAPTER 13		
Q#	Ans	Objective
1	A	13-1
2	C	13-2, 13-3, 13-4
3	C	13-2
4	B	13-3
5	A	13-3
6	D	13-8
7	A	13-4
8	C	13-5
9	C	13-6
10	C	13-6
11	C	13-7
12	C	13-9
13	D	13-9
14	A	13-10
15	B	13-10
16	C	13-11
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CHAPTER 14		
Q#	Ans	Objective
1	A	14-1
2	D	14-2
3	D	14-5
4	A	14-2
5	A	14-4
6	C	14-6
7	D	14-6
8	C	14-3
9	A	14-8
10	C	14-8
11	B	14-6
12	C	14-7
13	B	14-10
14	D	14-9
15	B	14-6
16	D	14-6

CHAPTER 15		
Q#	Ans	Objective
1	B	15-1
2	C	15-2
3	D	15-3
4	C	15-4
5	A	15-4
6	C	15-6
7	B	15-5
8	B	15-7

CHAPTER 16

Q#	Ans	Objective
1	A	16-1
2	C	16-4
3	A	16-3
4	B	16-2
5	C	16-3
6	D	16-5
7	D	16-5
8	A	16-7
9	C	16-4
10	A	16-6
11	D	16-8
12	A	16-10
13	B	16-13
14	C	16-12
15	C	16-7
16	B	16-13
17	D	16-6
18	C	16-9
19	A	16-11
20	C	16-13
21	C	16-14

CHAPTER 17

Q#	Ans	Objective
1	A	17-1
2	C	17-2
3	C	17-3
4	A	17-3
5	A	17-4
6	B	17-5
7	B	17-6
8	C	17-7
9	B	17-8
10	A	17-9

CHAPTER 18

Q#	Ans	Objective
1	C	18-7
2	B	18-3
3	D	18-7
4	C	18-11
5	A	18-7
6	B	18-6
7	D	18-6, 18-7
8	A	18-4
9	B	18-2
10	B	18-3
11	C	18-5
12	B	18-10
13	C	18-1
14	B	18-8
15	D	18-9

CHAPTER 19

Q#	Ans	Objective
1	B	19-1
2	B	19-2
3	B	19-4
4	C	19-4
5	D	19-5
6	B	19-6
7	C	19-6
8	A	19-6
9	C	19-7
10	C	19-6
11	C	19-8
12	B	19-9
13	A	19-10
14	D	19-11

CHAPTER 20

Q#	Ans	Objective
1	C	20-1
2	A	20-2
3	D	20-3
4	B	20-4
5	A	20-4
6	D	20-5
7	B	20-7
8	A	20-8
9	C	20-9
10	D	20-10
11	D	20-4
12	C	20-4
13	B	20-6

CHAPTER 21

Q#	Ans	Objective
1	C	21-1
2	B	21-2
3	C	21-2
4	C	21-3
5	A	21-4, 21-6
6	D	21-8
7	B	21-9, 21-14
8	B	21-10
9	C	21-11
10	D	21-7, 21-12
11	C	21-5, 21-13
12	A	21-13
13	B	21-2
14	C	21-9
15	D	21-9

CHAPTER 22

Q#	Ans	Objective
1	C	22-1
2	D	22-2
3	C	22-3
4	C	22-3
5	B	22-4
6	C	22-4
7	A	22-5
8	A	22-5
9	C	22-6
10	A	22-7
11	D	22-7
12	C	22-8
13	C	22-8
14	C	22-7
15	A	22-7
16	A	22-7
17	A	22-7
18	B	22-5
19	C	22-7
20	C	22-8
21	C	22-9
22	D	22-10
23	C	22-10

CHAPTER 23

Q#	Ans	Objective
1	C	23-9
2	A	23-9
3	A	23-4
4	A	23-11
5	B	23-9
6	C	23-9
7	D	23-9
8	A	23-8
9	C	23-1
10	B	23-2
11	B	23-2
12	D	23-2
13	C	23-2
14	B	23-2
15	C	23-3
16	A	23-3
17	C	23-4
18	B	23-5
19	B	23-6
20	C	23-7
21	D	23-8
22	C	23-9
23	B	23-9
24	C	23-10
25	B	23-11
26	D	23-11

CHAPTER 24

Q#	Ans	Objective
1	B	24-1
2	C	24-2
3	B	24-1, 24-3, 24-5
4	B	24-4
5	D	24-6
6	A	24-7
7	B	24-8
8	B	24-8
9	B	24-9
10	B	24-10
11	B	24-10
12	C	24-10
13	C	24-11
14	D	24-12
15	B	24-12
16	D	24-13
17	B	24-2, 24-3

CHAPTER 25

Q#	Ans	Objective
1	B	25-1
2	D	25-2
3	C	25-3
4	B	25-4
5	C	25-3
6	A	25-4
7	A	25-5
8	C	25-5
9	B	25-5
10	A	25-6
11	D	25-7
12	C	25-8
13	A	25-8
14	B	25-8
15	A	25-9
16	C	25-10
17	C	25-11
18	D	25-12
19	B	25-13
20	C	25-14

CHAPTER 26

Q#	Ans	Objective
1	B	26-1
2	A	26-2
3	C	26-2, 26-9, 26-10
4	B	26-2, 26-9
5	D	26-3
6	C	26-5, 26-6
7	A	26-8
8	C	26-11
9	B	26-4
10	C	26-7

CHAPTER 27

Q#	Ans	Objective
1	A	27-1
2	A	27-7
3	B	27-6
4	C	27-8
5	B	27-5
6	A	27-4
7	A	27-10
8	C	27-10
9	B	27-2
10	B	27-3
11	D	27-9

CHAPTER 28

Q#	Ans	Objective
1	C	28-2, 28-3
2	D	28-2, 28-3
3	D	28-10
4	C	28-2
5	B	28-2
6	C	28-2
7	C	28-8
8	A	28-2
9	B	28-9
10	D	28-2
11	B	28-1
12	C	28-4
13	C	28-5
14	B	28-6
15	A	28-7
16	C	28-9

CHAPTER 29

Q#	Ans	Objective
1	C	29-1
2	B	29-2
3	A	29-3
4	B	29-4
5	C	29-4
6	A	29-5
7	B	29-5
8	D	29-5
9	C	29-6
10	C	29-6
11	A	29-7
12	B	29-7
13	C	29-8
14	B	29-8
15	C	29-8
16	C	29-9
17	A	29-10
18	C	29-11
19	D	29-12
20	B	29-13

CHAPTER 30

Q#	Ans	Objective
1	C	30-2
2	D	30-11
3	B	30-8
4	D	30-9
5	C	30-1
6	C	30-3
7	B	30-5
8	D	30-6
9	C	30-7
10	A	30-4
11	D	30-10
12	B	30-12

CHAPTER 31

Q#	Ans	Objective
1	A	31-4
2	B	31-2
3	A	31-9
4	A	31-8
5	C	31-3
6	B	31-5
7	D	31-6
8	A	31-7
9	C	31-1

CHAPTER 32

Q#	Ans	Objective
1	A	32-3
2	C	32-2
3	B	32-6
4	A	32-2
5	C	32-2
6	B	32-2
7	C	32-2
8	B	32-2
9	C	32-1
10	B	32-1
11	C	32-1
12	B	32-1
13	D	32-4
14	A	32-5

CHAPTER 33

Q#	Ans	Objective
1	B	33-1
2	C	33-1
3	B	33-9
4	A	33-8
5	C	33-12
6	D	33-6
7	A	33-4, 33-5
8	A	33-13
9	A	33-14
10	C	33-2
11	D	33-3
12	A	33-6
13	B	33-7
14	C	33-10
15	A	33-11
16	D	33-12
17	B	33-15
18	B	33-16
19	C	33-17

CHAPTER 34

Q#	Ans	Objective
1	D	34-3
2	C	34-2
3	A	34-1
4	C	34-4
5	B	34-1

CHAPTER 35

Q#	Ans	Objective
1	B	35-6
2	A	35-5
3	C	35-6
4	B	35-7
5	A	35-3
6	C	35-1
7	A	35-8
8	D	35-6
9	A	35-9
10	D	35-8
11	A	35-8
12	C	35-9
13	B	35-9
14	C	35-2
15	D	35-9
16	A	35-4
17	C	35-10

A

- Absolute erythrocytosis:** A true increase in the number of circulating erythrocytes and hematocrit, as it happens in primary erythrocytosis (PV) and secondary erythrocytosis, related to high altitude, COPD, and smoking.
- Acanthocyte:** An abnormal red cell that is slightly reduced in size and that possesses 3 to 12 spicules of uneven length distributed along the periphery of the cell membrane.
- Accuracy:** A result that is close to the true value.
- Achlorhydria:** Absence of free hydrochloric acid in the stomach.
- Acrocyanosis:** Bluish tinge to the extremities.
- Activated partial thromboplastin time (aPTT):** A test to evaluate the overall integrity of the clotting system that involves factors XII, XI, IX, VIII, X, V, II, and I. Usually a means of evaluating the intrinsic system of coagulation.
- Activation:** Several separate responses of platelet function that include adhesion, shape change, secretion, and aggregation.
- Acute (decompensated) DIC:** A condition in which active hemorrhage is evident and the consumption of the coagulation factors and platelets exceeds the capacity to increase the synthesis of these components.
- Adhesion:** The molecular attraction exerted between the surfaces of bodies in contact (e.g., platelets to connective tissue structures).
- Adult T-cell leukemia:** Caused by human T-cell leukemia/lymphoma virus-1 (HTLV-1), with characteristic clinical features including generalized lymphadenopathy, hypercalcemia, bone and skin lesions, and 10% to 80% abnormal lymphoid cells in the blood and bone marrow.
- AE1:** The major integral membrane protein that performs an important transport function by regulating $\text{HCO}_3^-/\text{Cl}^-$ exchange and facilitating the transfer of carbon dioxide from tissues to lungs.
- Agglutination:** The clumping together of red blood cells or any particulate matter resulting from interaction of antibody and its corresponding antigen.
- Aggregation:** A clustering or clumping together (e.g., platelet aggregation, which plays a critical role in hemostasis).
- Alder-Reilly Inclusions (Alder's anomaly):** The presence of prominent, dark-staining, coarse cytoplasmic granules in neutrophils, eosinophils, basophils, monocytes, or occasionally lymphocytes.
- Allele:** In a particular person's genome, there are two copies of each gene, one inherited from mother and the other from father, representing the maternal and paternal alleles. Differences between the two alleles may be referred to as heterozygous polymorphisms, variants or mutations.
- A homozygous variant means that both alleles have the same variant that differs from a reference sequence.
- Alloantibody:** An antibody produced by an immune response that was stimulated by a foreign antigen.
- Allogeneic stem cell transplantation:** A transplant where the bone marrow stem cells from another individual with normal hematopoiesis are transplanted into the patient.
- Alloimmunization:** The process in which a patient develops antibodies to foreign or white blood cell antigen(s), or both, through transfusion or pregnancy.
- Amyloidosis:** A metabolic disorder marked by extracellular deposition of amyloid (an abnormal protein) in the tissues; this usually leads to loss of function and organ enlargement.
- Anaplastic large cell lymphoma (ALCL):** A relatively common form of mature T-cell lymphoma that represents a distinct clinical entity of young patients.
- Anemia:** A condition in which there is reduced oxygen delivery to the tissues. It may result from increased destruction of red cells, excessive blood loss, or decreased production of red cells.
- Aplastic anemia:** A type of anemia caused by aplasia of bone marrow or its destruction by chemical agents or physical factors.
- Hemolytic anemia:** A type of anemia caused by hemolysis of red blood cells resulting in reduction of normal red cell life span.
- Iron-deficiency anemia (IDA):** Anemia resulting from a greater demand on stored iron than can be met.
- Megaloblastic anemia:** Anemia in which megaloblasts are found in the blood; usually caused by a deficiency of folic acid or vitamin B12.
- Microangiopathic hemolytic anemia:** A hemolytic process associated with thrombotic thrombocytopenic purpura (TTP), prosthetic heart valve, and burns. It is visualized in the peripheral blood smear by fragmentation of the red cells and other bizarre morphology.
- Pernicious anemia:** A type of megaloblastic anemia caused by a deficiency of vitamin B12 that is directly linked to absence of intrinsic factor (IF).
- Sideroblastic anemia:** A disorder in which iron is not being incorporated into heme and serum iron levels are elevated. The bone marrow is hyperplastic and contains iron-laden sideroblasts.
- Anisocytosis:** Variation in the size of erythrocytes when observed on a peripheral blood smear.
- Ankyrin:** A pyramid-shaped protein that is a major component of the red cell cytoskeleton.
- Antibody titration:** A serial dilution of antibody conjugated fluorescent dyes. The purpose of performing titrations is to find the optimal amount of dye to add to a sample.

Adding too little dye may lead to false negative interpretations and adding too much may lead to non-specific, misleading staining.

Antihemophilic factor (AHF): A commercially prepared source of factor VIII.

Antiphospholipid Antibody Syndrome (APS): One of the most common causes of acquired coagulation defects associated with venous and/or arterial thrombosis.

Antithrombin (AT-III): A substance that opposes the action of thrombin and thus prevents or inhibits coagulation of blood.

Apoptosis: The natural process of programmed cell death.

Arthrocentesis: The procedure to obtain fluid from a joint space.

Articulations: The junctions between two or more bones.

Artifacts: Particulates that can appear in synovial fluid during microscopic analysis that are clinically insignificant and/or not originally of synovial origin. They must be differentiated from clinically significant synovial particulates.

Asynchrony: The failure of events to occur in time with each other as they usually do. In hematology, nuclear and cytoplasmic development are mismatched.

Auer rod: A rod-shaped alignment of primary granules that is present only in the cytoplasm of myeloblasts and monoblasts in leukemic states.

Autosplenectomy: Formation of a fibrotic, nonfunctioning spleen caused by restrictive blood flow to the organ; often seen in sickle cell anemia.

B

Base pair: The length of a DNA molecule is measured in base pairs (bp), or in thousands of base pairs = kilobases (kb).

Basophil: A mature white blood cell whose cytoplasmic granules stain deep bluish-purple with basic dyes such as methylene blue. It makes up 0% to 2% of the normal differential count.

Basophilic normoblast: An immature red cell precursor found only in the bone marrow that is characterized by a vivid blue cytoplasm and a high nuclear-to-cytoplasmic ratio. (Synonym: **Prorubricyte**.)

Benchmarking: The practice of comparing the individual laboratory processes and performance metrics to industry best practices from other laboratories.

Bernard-Soulier syndrome (BSS): A congenital bleeding disorder characterized by the presence of large platelets, thrombocytopenia of varying degrees, and a prolonged bleeding time.

Bias: A quantitative term describing the difference between the average of measurements made on the same object and its true value.

Bilirubin: The orange or yellowish pigment in bile that is carried to the liver by the blood. It is produced from hemoglobin of red blood cells by reticuloendothelial cells in the bone marrow, spleen, and elsewhere.

Indirect bilirubin: The unconjugated water-insoluble form of bilirubin.

Birefringence: The ability of a particular material to refract light.

Bite cells: Cells in which the removal of a portion of membrane has left a permanent indentation in the remaining cell membrane.

Bleeding time test: A test used to evaluate the hemostatic role of platelets in vivo.

Blister cell: A cell that escapes from the fibrin strand, appearing in the peripheral blood as a red cell with a vacuole in one end resembling a blister.

Burr cells (echinocytes): Red cells with approximately 10 to 30 spicules evenly distributed over the surface of the cell.

C

Cabot's rings: A red blood cell inclusion resembling a figure 8. It is usually found in heavily stippled cells.

Calmodulin: A cytoplasmic calcium-binding protein.

Cardiac tamponade: A severe complication of pericarditis or caused by a traumatic injury. Occurs when pericardial fluid or blood within the pericardial space, under increased pressure, restricts the motion of the heart.

Cardiolipin: An anionic phospholipid found in the inner mitochondrial membranes of cardiac and skeletal muscle cells and some bacteria; known as diphosphatidylglycerol.

Catastrophic antiphospholipid syndrome (CAPS): A serious and often fatal manifestation of APS characterized by the development of multiorgan thromboses (infarctions) over a very short period of time (days to weeks).

cDNA (complementary DNA): Synthetic DNA transcribed from an RNA template by the enzyme reverse transcriptase.

Cerebrospinal fluid (CSF): A selective ultrafiltrate of plasma that protects and supports the brain and spinal cord and maintains a constant ionic environment by circulating nutrients and removal of waste products.

Chelation: Combining of metallic ions with certain hetero-cyclic ring structures so that the ion is held by chemical bonds from each of the participating rings.

Chemokinesis: Increased activity of cells in the presence of a chemical attractant.

Chemotaxis: Describes movement toward a stimulus, particularly that displayed by phagocytic cells toward bacteria and sites of cell injury.

Choroid plexus: Masses of specialized capillaries in the pia mater.

Classic Hodgkin lymphoma: Represents the majority of cases of Hodgkin lymphoma cases and is morphologically defined by classic-appearing Reed-Sternberg cells.

Cluster of Differentiation (CD): CD molecules are cell surface markers that are useful for the characterization and identification of leukocytes and their subpopulations.

Cobalamin: Vitamin B12; a large, water-soluble molecule.

Codocyte: From the Greek word "kodon" meaning bell. See **Target cells**.

Coefficient of variation (CV): Describes the SD as a percentage of the mean.

Cofactor: A factor that facilitates binding and accelerates enzymatic interaction of other coagulation factors with their target protein or substrate on an active biologic surface.

Colony-stimulating factors (CSFs): Produced by T lymphocytes—as well as stromal cells, fibroblasts, endothelial cells, and macrophages—when stimulated by monocyte interleukin-1 (IL-1) and tumor necrosis factor (TNF).

Complement: A series of proteins in the circulation that, when sequentially activated, cause disruption of bacterial and other cell membranes. Activation occurs via one of two pathways, and once activated, the components are involved in a great number of immune

Consanguineous: Relationship by blood (i.e., being descended from a common ancestor).

Continuous ambulatory peritoneal dialysis (CAPD): A procedure that utilizes the natural properties of the peritoneal membrane and the infusion of a dialyzing fluid into the cavity to remove impurities in the bloodstream.

Control chart: A graphical method to display control results so that it is easier to determine whether a control run is in or out-of-control. Shifts and trends can be visually assessed using control charts.

Control limits: Statistical criteria of acceptability for a particular control material, usually derived from the mean and standard deviation (SD). The limits are placed on a control chart for ease of use.

Control rule: A decision criterion for judging a control material as acceptable or not. Westgard MultiRules are examples of control rules.

Cytokines: Growth factors, such as colony-stimulating factors and interleukins.

Cytopenia: Abnormalities or deficiencies in blood cell elements.

D

Dacryocytes: See **Teardrop cells**.

Define, Measure, Analyze, Improve, and Control (DMAIC): A five-phase method for defining the problem and improving the activity

Delta checks: Comparison of the difference between measurements of an analyte (or combinations of analytes) on two separate samples from the same patient to predefined thresholds representing the limits of acceptable change.

Diapedesis: The journey of the blood cells (i.e., leukocytes) through the unruptured walls of a capillary.

Diffuse basophilic stippling: Stippling that appears as a fine blue dusting.

Dimer: A compound formed by the combination of two identical molecules.

D-dimer: Degradation of cross-linked fibrin generated by plasmin.

Dimorphic population: A mixture of large and small cells in case of a normal MCV and a high RDW (normal RDW is 11.5% to 14.5%).

Disseminated intravascular coagulation (DIC): A pathologic form of coagulation that is systematic

rather than localized, and is characterized by generalized bleeding and intravascular clotting.

Distribution: How cells are dispersed on the peripheral smear slide.

Divalent Metal Transporter 1: Protein responsible for iron transport protein from gastrointestinal lumen into the duodenal enterocyte and from the erythroblast endosome into the cytoplasm.

DNA (deoxyribonucleic acid): The blueprint that cells use to catalog and express information and to propagate it from one generation to the next. It is a double-stranded molecule composed of complementary nucleotide sequences. The strands are held together by hydrogen bonds formed according to the rules of complementary nucleotide pairing: G bonds with C, A bonds with T.

DNA sequencing: a laboratory procedure to determine the order of nucleotides within DNA.

Döhle bodies: Single or multiple, round or oval, blue cytoplasmic inclusions (with Romanowsky stain) seen in neutrophils, usually associated with toxicity.

Döhle body-like: Oval, blue, single, or multiple cytoplasmic inclusions originating in RNA and 1 to 3 μm in diameter. Characteristically observed in certain congenital qualitative WBC disorders such as May-Hegglin anomaly and Chédiak-Higashi disorder.

Donath-Landsteiner test: A test usually performed in the blood bank to detect the presence of the Donath-Landsteiner antibody, which is a biphasic IgG antibody with anti-P specificity found in patients suffering from paroxysmal cold hemoglobinuria.

Drepanocytes: Cells also known as sickle cells that are typically crescent- or sickle-shaped with pointed projections at one or both ends of the cell. These cells have been transformed by hemoglobin polymerization into rigid, inflexible cells no longer resembling the normal biconcave disc.

Dyserythropoiesis: Changes in erythroid cell nuclear chromatin pattern; some of these changes are bizarre.

Dyspnea: Labored or difficult breathing.

E

Echinocytes: See **Burr cells**.

Elliptocyte: Pencil-shaped cells, invariably not hypochromic.

Elution: A process whereby cells that are coated with antibody are treated in such a manner as to disrupt the bonds between the antigen and the antibody. The freed antibody is collected in an inert diluent such as saline or 6% albumin. This serum can then be tested to identify its specificity using routine methods. The mechanism to free the antibody may be physical (heat, shaking) or chemical (ether, acid), and the harvested antibody-containing fluid is called an *eluate*.

Empyema: The collection of pus in the pleural cavity.

Enterocyte: Epithelial cell that lines the small and large intestine.

Enzyme-linked immunosorbent assay (ELISA): A method that utilizes a microtiter plate coated with specific rabbit antihuman vWF antibodies to capture the vWF to be measured.

Eosinophil: A mature type of granulocyte in which cytoplasmic granules are large, round, and refractile and stain orange or red with Wright's stain. It comprises 0 to 4% of the normal differential count.

Error: The difference between the true value and the obtained value.

Erythroferrone (ERFE): Hormone produced by erythroblasts that suppresses hepcidin.

Erythropoiesis: The production and maturation of erythrocytes.

Essential thrombocythemia (ET): A rare but serious myeloproliferative neoplasm (MPN) characterized by marked thrombocytosis with bone marrow megakaryocytic hyperplasia and a tendency to develop thrombotic and hemorrhagic complications.

Euglobulin lysis time (ELT): A coagulation procedure testing for fibrinolysins.

Extramedullary hematopoiesis: Formation of blood cells in sites other than the bone marrow (i.e., liver, spleen).

Exudates: Effusions that accumulate due to a primary pathological state within the compartment.

Extravascular hemolysis: Hemolysis occurring within the cells of the reticuloendothelial system.

F

Fatty yellow marrow: A dynamic tissue similar to hematopoietic tissue that varies in amount according to the age of the patient and the skeletal location from where the marrow is obtained. Only a few fat cells are present in young children, where as in adults, fat cells average about 50% of total marrow volume in the vertebrae and flat bones of the pelvis.

Favism: An inherited condition resulting from sensitivity to the fava bean, usually seen in people of Mediterranean origin who have a deficiency in the enzyme glucose-6-phosphate dehydrogenase, which may result in a severe hemolytic episode.

Fenestrations: Openings or "windows" between the cytoplasmic membranes of the cells.

Ferritin: The storage form of iron in the tissues, found principally in the reticuloendothelial cells of the liver, spleen, and bone marrow.

Ferrochelatase: Enzyme that catalyzes the insertion of iron into protoporphyrin IX to form hemoglobin.

Ferroportin: Protein that transports iron from enterocytes and macrophages into the bloodstream.

Fibrin: A whitish filamentous protein or clot formed by the action of thrombin on fibrinogen, converting it to fibrin.

Fibrin-forming (coagulation) system: The system through which coagulation factors interact to eventually form a fibrin clot.

Fibrin monomer: The altered molecule that results from thrombin splitting fibrinopeptides A and B from two of the three paired chains of the fibrinogen molecule.

Fibrinogen degradation products (FDPs): The polypeptide fragments X, Y, D, and E that result from the proteolytic action of plasmin on fibrinogen or fibrin.

Fibrinogen group: Consists generally of high molecular weight proteins that include factors I (fibrinogen), V (labile factor), VIII (antihemophilic factor), and XIII (fibrin-stabilizing factor).

Fibrinolysis: Dissolution of fibrin by fibrinolysin, caused by the action of proteolytic enzyme system that is continually active in the body but increased greatly by various stress stimuli.

Fluorescence Compensation: A mathematical algorithm that removes fluorescence spillover of one fluorophore into multiple detectors.

Fluorochromes: (or fluorophores) are dyes that absorb, and then emit, light which produces a color. These dyes are coupled with antibodies to surface and intracellular markers. Cells stained with fluorescent antibody conjugate travel single-file through a flow cell on a flow cytometer and the fluorochrome is excited by light. The light signal is measured and evaluated.

Follicular lymphoma: A form of non-Hodgkin lymphoma that has a mixture of lymphocytes, mainly centrocytes (small cleaved) and centroblasts (large noncleaved) cells that have a B-cell immunophenotype (CD19+, CD20+, CD5-) and t(14;18) *IGH-BCL2*.

G

Gastrectomy: Surgical removal of part or all of the stomach.

Gating: Selecting an area on a scatter plot which contains the population of cells to be further analyzed.

Gaucher's disease: A familial, lysosomal disorder caused by a deficiency in the enzyme beta-glucocerebrosidase.

Gene: A functional segment of DNA that serves as a template for RNA transcription and protein translation. Regulatory sequences control gene expression, so that only a small fraction of the estimated 100,000 genes are ever transcribed by a given cell.

Gene rearrangement: A process in which segments of DNA are cut and spliced to produce new DNA sequences. During normal lymphocyte development, rearrangement of the immunoglobulin genes and the T cell receptor genes results in new gene sequences that encode antibody and surface antigen receptor proteins necessary for immune function.

Genome: The total aggregate of inherited genetic material. In humans, the genome consists of 3 billion base pairs of DNA divided among 46 chromosomes, including 22 pairs of autosomes numbered 1-22, and two sex chromosomes (XX or XY). Each type of microbe has a species-specific genome comprised of either DNA or RNA.

Genotype: The genetic constitution of a particular specimen.

Ghrelin: A hunger hormone released by the stomach.

Glanzmann's thrombasthenia: A congenital bleeding disorder characterized by impaired or absent clot retraction and a failure of the platelets to aggregate with most aggregating agents, particularly with ADP.

Globin: A protein constituent of hemoglobin. There are four globin chains in the hemoglobin molecule.

Glossitis: Inflammation of the tongue.

Glucose-6-phosphate dehydrogenase (G6PD): An intracellular red cell enzyme important in the hexose monophosphate pathway.

Glycophorin: The principal integral blood cell protein, containing 60% carbohydrate and giving the red cell its negative charge. It appears on the external surface of the red cell.

Gout: A hereditary metabolic disease that is a form of acute arthritis and is marked by inflammation of the joints. The affected joint may be at any location, but gout usually begins in the knee or foot.

Granulocyte: A white blood cell with small granules that rids the body of infection or allergens; refers to band or polymorphonuclear neutrophil, eosinophil, or basophil.

Granulopoiesis: The production and maturation of granulocytes.

H

Hairy cell leukemia (HCL): A rare indolent neoplasm of mature lymphocytes with oval to kidney-shaped nuclei and abundant cytoplasm and "hairy" cell surface projections.

Haptoglobin: An 2-glycoprotein produced in the liver, having three phenotypes with differing abilities to bind hemoglobin.

Heinz bodies: Large red blood cell inclusions that are formed as a result of denatured or precipitated hemoglobin. May be seen in the thalassemia syndromes, G6PD deficiency, or any of the unstable hemoglobin conditions.

Helmet cells: See **Blister cells**.

Hemarthrosis: Bloody effusion into the cavity of a joint.

Hematogones: Normal cellular constituents of bone marrow that resemble small- to intermediate-sized lymphocytes.

Hematoidin crystals: A product of hemoglobin catabolism appearing as bright yellow or red crystals.

Hematolymphoid disorders: Bening conditions and malignant diseases related to lymphoid cells.

Hematopoiesis: Formation and development of blood cells, normally in the bone marrow. (Synonym: **Hemopoiesis**.)

Hematuria: Blood in the urine.

Hemochromatosis: A disease of iron metabolism in which iron accumulates in body tissues, causing complications and tissue damage.

Hemoconcentration: An increase in the number of red cells, resulting from a decrease in the volume of plasma.

Hemoglobin (Hb) C crystals: Found in hemoglobin C disease—a mild chronic hemolytic anemia in which the patient is homozygous for the abnormal hemoglobin C.

Hemoglobin SC (HbSC) crystals: Found on the peripheral smears of patients diagnosed with HbSC disease—a chronic hemolytic disorder punctuated by acute painful crisis and diverse chronic organ damage, secondary to the presence of both HbS and HbC.

Hemoglobinemia: Presence of hemoglobin in the blood plasma.

Hemoglobin-oxygen dissociation curve: The relationship between the % saturation of the hemoglobin molecule with oxygen and the environmental oxygen tension.

Hemoglobinuria: The presence of free hemoglobin in the urine.

Hemolysis: The destruction of red blood cells.

Intravascular hemolysis: The disruption of the red cell membrane and release of hemoglobin into the surrounding fluid within the vasculature.

Extravascular hemolysis: The phagocytosis of erythrocytes by the reticuloendothelial system, primarily in the spleen and liver.

H. disease of the fetus and newborn (HDFN): A disease characterized by anemia, jaundice, enlargement of the liver and spleen, and generalized edema (hydrops fetalis), and caused by maternal IgG antibodies that cross the placenta and attack fetal red cells when there is a fetomaternal blood group incompatibility. Usually caused by ABO or Rh antibodies. (Synonym: **Erythroblastosis fetalis**.)

Hemopexin: A beta globulin that has the capacity to bind hemoglobin when haptoglobin has been depleted.

Hemophilia: A hereditary blood disease characterized by impaired coagulability of the blood and a strong tendency to bleed.

H. A: A sex-linked hereditary bleeding disorder characterized by greatly prolonged coagulation time, owing to a deficiency of factor VIII.

H. B: Christmas disease, a hereditary bleeding disorder caused by a deficiency of factor IX.

H. C: A hereditary bleeding disorder caused by a deficiency of factor XI.

Hemosiderin: An iron-containing pigment derived from hemoglobin on disintegration of red cells; one method whereby iron is stored until needed for making hemoglobin.

Hemothorax: Pure blood in the pleural cavity that can result from severe chest injuries, stab or gunshot wounds, or surgical procedures.

Hepatomegaly: A condition characterized by enlargement of the liver.

Hepcidin: Iron-regulating protein and acute phase reactant produced by the liver. It acts to decrease iron absorption from the small intestine and block iron release from macrophages. This reduces the availability of iron for hematopoiesis and is important in the development of anemia of chronic disease.

Hephaestin: Copper dependent enzyme that oxidizes iron to facilitate its transport by ferroportin across enterocyte membrane into bloodstream.

Hereditary hemochromatosis (HH): A recessive genetic disorder. It is one of the most frequent genetic diseases in North America, typically found in those with northern European ancestry, affecting approximately 1 in 300 people.

Hereditary spherocytosis (HS): An inherited (autosomal-dominant) intracorpuseular defect of the red cell membrane

(altered spectrin) that results in the most common hereditary hemolytic anemia found in whites. The morphological hallmark of hereditary spherocytosis is the presence of spherocytes on the peripheral blood smear.

Hereditary stomatocytosis (hereditary hydrocytosis): A heterogeneous group of rare red cell membrane disorders inherited in an autosomal-dominant fashion that are characterized by the presence of stomatocytes on the peripheral blood smear and alterations in the permeability of the red cell membrane to cations.

Hirudin: An anticoagulant first discovered in leeches, now manufactured with recombinant methods, that anticoagulates by directly inhibiting thrombin and blocking its ability to convert fibrinogen to fibrin. It includes varieties lepirudin, desirudin, and bivalirudin.

Howell-Jolly bodies: Red cell inclusions that develop in periods of accelerated or abnormal erythropoiesis. They represent nuclear remnants containing DNA.

Hybridization: The process by which one nucleotide strand binds to another strand by forming hydrogen bonds between complementary nucleotides.

Hydrocytosis: An increase in red cell hydration and volume due to abnormalities in red cell cation permeability.

Hydrodynamic focusing: Hydrodynamic focusing is accomplished by a flow cytometer's fluidic system. Cells suspended in a core stream are guided through the flow cell by sheath fluid. Due to the pressure differences between the sample stream and sheath, the two liquids do not mix. Depending on the cell concentration of the sample the flow rate has to be adjusted to avoid more than one cell's passing through the flow cell at the same time.

Hyperchromic: Red cells with a decreased surface-to-volume ratio and a decreased or absent central pallor.

Hyperproteinemias: Abnormally high levels of protein in your blood plasma.

Hypersegmentation: An increase in the number of nuclear lobes or segments (more than 5) in segmented neutrophils; especially characteristic of vitamin B12 or folate deficiencies.

Hypochromic: The term means "low color" and indicates that the cells have less than the normal amount of hemoglobin.

Hypoplasia: Refers to abnormal, deficient, or defective development.

Hyposegmentation: A decreased segmentation consisting of neutrophils with two lobes or less.

I

Immunoblast: A mitotically active T or B cell.

Immunoglobulin (Ig): One of a family of closely related, yet not identical, proteins that are capable of acting as antibodies: IgA, IgD, IgE, IgG, and IgM. The principal immunoglobulin in exocrine secretions such as saliva and tears is IgA. IgD may play a role in antigen recognition and the initiation of antibody synthesis. IgE is produced by the cells lining the intestinal and respiratory tracts and is important in forming reagin. The main immunoglobulin

in human serum is IgG. A globulin formed in almost every immune response during the early period of the reaction is IgM.

Immunophenotyping: A flow cytometric laboratory method that identifies cell surface and intracellular markers in order to characterize cells and establish diagnoses.

In situ follicular neoplasia (ISFN): Germinal centers are partially or completely colonized by clonal B cells carrying the BCL2 translocation characteristic of follicular lymphoma.

In situ hybridization: Localize DNA or RNA by probe binding in cells, tissue or chromosomes immobilized on a glass slide. In a variation of this procedure called fluorescence *in situ* hybridization (FISH), a fluorochrome attached to the probe permits microscopic visualization of the target nucleic acid in cells or in metaphase chromosomes.

Integral membrane proteins: Proteins that extend from the outer surface and traverse the entire membrane to the inner cytoplasmic side of the RBC.

Interference experiment: Provides information about the constant systematic error (accuracy, bias) caused by interfering substances.

Internal assessments: Internal assessments include correlations between similar instruments, audits, self-inspections, and quality monitors. These assessments examine how the laboratory is meeting its own requirements.

Interrogation point: The location in the flow cell where the laser intersects the sample.

Intravascular hemolysis: Hemolysis occurring within the blood vessels. (See Hemolysis.)

Intrinsic factor (IF): A protein secreted by the parietal cells of the stomach that is necessary for vitamin B12 absorption.

J

JAK2: Janus kinase 2, the most common mutation found with MPNs which is located on chromosome 9q24 and is frequently found in ~95% of PV and 60% of ET and PMF, among other disorders such as RARS-T (refractory anemia with ring sideroblasts associated with marked thrombocytosis).

Joint capsule: Strong fibrous tissue that encloses freely movable limb joints of the body for support and alignment of the bones.

K

Karyorrhexis: A necrotic stage with fragmentation of the nucleus, whereby chromatin is distributed irregularly throughout the cytoplasm.

Keratocytes: See Bite cells.

L

Laboratory Developed Tests (LDTs): In vitro diagnostic tests that are designed, validated and used within a single laboratory.

Lacunar cell: A distinctive Reed-Sternberg variant separated from surrounding cells by a large clear space (a lacuna) located in a sea of lymphocytes, plasma cells, eosinophils, and neutrophils.

Lavage: Flushing of the peritoneal space with normal saline solution.

Lean: A quality plan that reduces waste in order to increase value in customers.

Leukemia: A chronic or acute disease of unknown etiologic factors characterized by unrestrained growth of leukocytes and their precursors in the tissues.

Leukemoid reaction: A moderate or advanced degree of leukocytes in the blood that is not a result of a leukemic disease. These reactions are frequently observed as a feature of infectious disease, drug and chemical intoxication, or secondary to nonhematopoietic carcinoma.

Leukocytosis: An increase in number of leukocytes (more than 10,000 cells per cubic millimeter) in the blood.

Littoral cells: Cells located in the walls of blood sinuses or lymph, characterized by their flattened appearance.

Locus: A specific position on a chromosome.

Lymphocyte: A white blood cell formed in lymphoid tissue throughout the body, generally described as nongranular and including small and large varieties. It makes up approximately 20% to 45% of the total leukocyte count.

Lymphocyte-rich Hodgkin lymphoma (HL): Characterized by a background rich in lymphocytes, in which the Reed-Sternberg cells exhibit the histologic appearance and immunophenotype of classic Reed-Sternberg cells rather than the popcorn cells seen in NLPHL.

Lymphoma: Asymmetric enlargement of a group of lymph nodes, which destroys the normal histologic lymph node architecture.

Lymphoplasmacytic lymphoma: A cancer of older adults in which the neoplastic lymphocytes exhibit plasmacytoid features and express monoclonal surface and cytoplasmic immunoglobulin, usually of the IgM class.

Lymphopoiesis: Refers to the growth or development of lymphocytes.

M

Macrocyte: A red cell 9 μm in diameter or larger.

Macrocytic: Anemia associated with macrocytes that is classified as either megaloblastic or nonmegaloblastic.

Macrocytosis: Refers to a condition in which erythrocytes are abnormally large.

Macro-ovalocytes: Refers to a condition in which ovalocytes are abnormally large.

Macrophage: A cell of the reticuloendothelial system having the ability to phagocytose particulate substances and to more vital dyes and other colloidal substances; found in loose connective tissues and various organs of the body.

Marginal zone lymphoma (MZL): Typically an extranodal clonal proliferation of mucosa-associated lymphoid tissue (MALT-lymphoma) at sites such as stomach, salivary gland, lung, thyroid, or orbit.

Matrix: A term describing the substance of the QC material. Regulations require that the control material be as close

as possible to real human specimens. For example, urine tests should be controlled by a urine-like control material, etc. Sometimes animal materials are used because they are very close in nature to the human materials and easier to manufacture.

Maturation storage pool: Consists of nondividing metamyelocytes, bands, and segmented neutrophils. The cells typically spend 5 to 7 days in this pool before entering into the circulation.

May-Hegglin anomaly: Inclusions found in the hereditary leukocyte and platelet disorder; similar, but not identical to, Döhle bodies.

Mean: The average of a set of numbers.

Megakaryopoiesis: The development of megakaryocytes in the blood.

Megaloblast: A large, nucleated, abnormal red cell precursor, 11 to 20 μm in diameter, oval and slightly irregular, resulting from a nuclear-cytoplasmic maturation synchrony characteristic of vitamin B12 or folate deficiency.

Megaloblastic: Erythropoiesis leading to fewer cellular divisions and consequently a larger cell.

Melena: Black tarry feces caused by the action of intestinal juices on free blood.

Melanocyte: A pigment-producing cell.

Mesothelial cells: A single layer of mesodermal cells that line the pericardial sac, forming the pericardium.

Methemoglobin (MetHgb): A form of hemoglobin wherein the ferrous ion (Fe^{2+}) has been oxidized to ferric ion (Fe^{3+}), possibly owing to toxic substances such as aniline dyes, potassium chlorate, or nitrate-contaminated water.

Method validation: A series of test and calculations performed to validate each parameter of a new method.

Microcyte: An abnormally small red cell with a diameter of less than 6 μm .

Microcytic: Anemia associated with microcytes resulting in an increase in cellular divisions and consequently a smaller cell in the peripheral blood.

Microspherocytes: Small, sphere-shaped red blood cells seen in certain kinds of anemia.

Mixed cellularity Hodgkin lymphoma (HL): Characterized by Reed-Sternberg cells surrounded by a heterogeneous mixture of cells including lymphocytes, histiocytes, plasma cells, and eosinophils.

Monocyte: A white blood cell that normally constitutes 2% to 10% of the total leukocyte differential count. This cell is 9 to 12 μm in diameter and has an indented nucleus and an abundant pale bluish-gray cytoplasm containing many fine red-staining granules.

Mononuclear phagocyte system (MPS): Formerly called the *reticuloendothelial system*: a system of mononuclear phagocytic cells scattered throughout the body. It includes monocytes and macrophages in the blood and bone marrow, histiocytes of loose connective tissue, reticular cells of lymphatic organs, Kupffer cells of the liver, cells lining blood sinuses of the spleen, and others.

Morulae: Intracellular clumps of blue-grey stained bacteria found in the cells of patients with Human Granulocytic Anaplasmosis.

Moving averages: Researchers discovered that red blood cell indices (mean corpuscular volume [MCV], mean corpuscular hemoglobin [MCH], and mean corpuscular hemoglobin concentration [MCHC]) were stable in a normal person over a long period of time.¹³ The mean of the RBC indices in the population of a laboratory are monitored for significant drifts or shifts in calibration, which may mean method quality problems. This QC procedure should not be the only source of QC of RBC indices, and laboratories running fewer than 100 CBCs a day should not utilize this procedure due to lack of sufficient data.

Mucopolysaccharidoses (MPS): A group of hexosamine-containing polysaccharides that are the major constituent of mucus.

Multiple myeloma: A neoplastic proliferation of plasma cells, characterized by very high immunoglobulin levels of monoclonal origin.

Mutation: A change in the nucleotide sequence of DNA. When a large series of nucleotides are missing, the alteration is referred to as a *deletion* or more generally as a *gene copy number variant*.

Myelodysplastic syndrome (MDS): A group of primary hematologic disorders associated with abnormal division, maturation, and production of erythrocytes, granulocytes, monocytes, and platelets; also referred to as *preleukemic myelodysplastic syndrome*.

Myeloid-to-erythroid (M:E) ratio: A differential count of bone marrow obtained by dividing the number of granulocytes and their precursor cells by the number of nucleated red cells.

Myelophthisic: The process that occurs primarily in the bone marrow as a result of the crowding out of normal elements by malignant cells. A consequential reduction in normal marrow cells and release of immature hematopoietic cells (especially nucleated red cells) into the blood occurs.

Myelopoiesis: The growth or development of myeloid cells in the bone marrow.

Myeloproliferative disorders: Also called chronic myeloproliferative disorders, this refers to a group of related neoplastic disorders including chronic myelogenous leukemia (CML), polycythemia vera, essential thrombocythemia, and chronic idiopathic myelofibrosis. All are characterized by autonomous proliferation of one or more bone marrow elements accompanied by enlargement of the spleen.

N

Neutropenia: The presence of abnormally small numbers of neutrophils in the circulating blood.

Neutrophilia: An abnormal increase in neutrophil leukocytes.

Nonmegaloblastic: Accelerated erythropoiesis, resulting from conditions such as acute blood loss or alcoholism.

Normochromic: Indicates the red cell is essentially normal in color. A normochromic erythrocyte has a

well-hemoglobinized cytoplasm with a small but distinct zone of central pallor. Area of pallor does not exceed 3 μm when measured linearly.

Normocyte: Having a normal erythrocyte.

Normocytic: Anemia with normal MCHC and MCH in conjunction with normal MCV.

Nuclear molding: The process whereby the nucleus of one cell molds around the shape of an adjacent cell; occurring with the cohesive growth of tumor cells that require the presence of tight junctions between the cytoplasmic membranes of the cells.

Nucleotide: The basic building block of DNA, composed of a nitrogen base (A=adenine, T=thymine, G=guanine, or C=cytosine) attached to a sugar (deoxyribose) and phosphate.

O

Oncogene: A gene that contributes to the development of cancer. Most oncogenes are activated forms of normal genes that function to stimulate cell growth.

Orthochromic (orthochromatophilic) normoblast: An immature red cell precursor characterized by pink cytoplasm and a small, round, pyknotic nucleus. This stage of maturation is normally found only in the bone marrow.

Osmotic fragility: The ability of the red cells to withstand different salt concentration; this is dependent on the volume, surface area, and functional state of the red blood cell membrane.

Osteoblast: An immature bone marrow cell responsible for the formation of osteocytes.

Osteoclast: A giant multinuclear cell formed in the bone marrow of growing bones.

Ovalocyte: An abnormal red cell that is egg-shaped or elliptical.

Oxyhemoglobin: The combined form of hemoglobin and oxygen.

P

Pancytopenia: A depression of each of the normal bone marrow elements: white cells, red cells, and platelets in the peripheral blood.

Pappenheimer bodies: Basophilic inclusions in the red blood cell that are cluster-like. They are believed to be iron particles; confirmation is made by Prussian blue stain.

Parachromatin: The portions of the nuclear chromatin that are unstained or lightly stained.

Parenteral: Entry into the body through the intravenous (IV) or intramuscular (IM) route rather than the alimentary route.

Paresthesia: Numbness.

Paroxysmal cold hemoglobinuria (PCH): A type of cold autoimmune hemolytic anemia usually found in children suffering from viral infections in which a biphasic IgG antibody can be demonstrated with anti-P specificity. (See also **Donath-Landsteiner antibody test**.)

- Paroxysmal nocturnal hemoglobinuria (PNH):** An uncommon acquired form of hemolysis caused by an intrinsic defect in the red blood cell membrane, rendering it more susceptible to hemolysins in an acid environment, and characterized by hemoglobin in the urine following periods of sleep.
- Pelger-Huët anomaly:** A benign hereditary condition possibly indicated by decreased segmentation.
- Pericardial cavity:** The potential space between the two membranous surfaces, containing a small amount of serous fluid that is secreted by the parietal pericardium.
- Pericardial effusion:** The abnormal accumulation of fluid in the pericardial space.
- Pericardial sac:** Fibrous structure the heart is enclosed within.
- Pericardiocentesis:** A procedure that removes pericardial fluid built up within the pericardial space.
- Pericardium:** The membrane that covers the parietal (inner) surface of the pericardial sac and the visceral (outer) surface of the heart.
- Peritoneum:** A single layer membrane of mesothelial cells that lines the abdominal cavity.
- PFA-100® closure time:** Utilizes optical aggregometry as a screening test for platelet function and is performed on citrated whole blood. It evaluates the ability of platelets to interact with collagen and ADP or epinephrine in a capillary setting to close the lumen while under flow conditions similar to that of the capillary vasculature.
- Phagocyte:** A cell that ingests foreign particles, microorganisms, or other cells.
- Phenylhydrazine:** A strong oxidizing agent.
- Pia mater:** An inner membrane that lies directly on the surface of the brain and spinal cord.
- Plasma cell:** A B lymphocyte-derived cell that secretes immunoglobulins or antibodies.
- Plasmacyte:** A plasma cell.
- Plasmin:** A fibrinolytic enzyme derived from its precursor, plasminogen.
- Plasminogen:** A protein found in many tissues and body fluids. It is important in preventing fibrin clot formation.
- P. activators:** Endothelial cell-derived plasma proteins, which convert plasminogen to plasmin.
- P. activator inhibitors-1 (PAI-1):** Endothelial cell proteins, which bind to tissue plasminogen activator (t-PA) and urokinase to inhibit their actions.
- Platelet (PLT):** A round or oval disc, 2 to 4 μm in diameter, derived from the cytoplasm of the megakaryocyte, a large cell in the bone marrow. Plays an important role in blood coagulation, hemostasis, and blood thrombus formation.
- Platelet aggregation:** Platelet-to-platelet interaction, dependent on calcium.
- Platelet-derived growth factor (PDGF):** A hematopoietic growth factor that stimulates fibroblasts to divide and secrete collagen.
- Pleocytosis:** The presence of more than a normal number of cells, particularly WBC, in CSF.
- Pleura:** A thin membrane composed of a single layer of mesothelial cells encasing the lungs.
- Pleural cavity:** The potential space between the visceral pleura and the parietal pleura that contains a thin film of serous fluid produced by the parietal pleura and absorbed by the visceral pleura.
- Pleural effusion:** Fluid in the pleural space.
- Pluripotent stem cells:** A generalized parent cell that gives rise to a common lymphoid stem cell, which differentiates into T- or B-cell ontogeny or a colony forming unit (CFU).
- Poikilocytic:** Erythrocytes that vary only slightly from the concave round shape of a biconcave disc, taking on such peculiar shapes as teardrops, pencils, and sickles.
- Poikilocytosis:** Variation in shape of red cells.
- Polychromatophilic:** RBCs delivered to the peripheral circulation prematurely that appear gray-blue (diffusely basophilic) in color and usually larger than normal red cells.
- Polychromatophilic normoblast:** An immature red cell precursor characterized by bluish-gray cytoplasm and a round, eccentrically located nucleus with a distinct chromatin/parachromatin pattern of staining, and normally only found in the bone marrow. (Synonym: **Rubricyte**.)
- Polymerase chain reaction (PCR):** A method of enzymatically amplifying (copying) a particular segment of DNA through a process of repeated cycles of heating, cooling, and DNA synthesis. To accomplish this, patient DNA is mixed with the chemicals needed for DNA synthesis (including deoxyribonucleotide "building blocks", the enzyme *DNA polymerase* that copies single stranded DNA to form double-stranded DNA, and two short DNA probes called primers that are designed to span the particular segment of DNA that will be amplified). A thermocycler instrument is programmed to sequentially heat and cool the sample. In cycle #1, the sample is heated to 95°C to dissociate complementary strands of DNA, then cooled to 55°C to permit binding of the short DNA probes that serve as primers for enzymatic DNA replication at 72°C. This replication generates new complementary strands that represent an exact copy of the original target DNA. In subsequent cycles, the products of previous cycles serve as templates for DNA replication, allowing an exponential accumulation of DNA copies. After 30 cycles, which takes only a couple of hours, approximately a billion copies of each target DNA have been produced. This permits rapid, sensitive, and specific identification of a segment of DNA that can then be further evaluated for a disease-specific genetic defect.
- Porphyrias:** A group of inherited disorders caused by excessive production of porphyrins in the bone marrow or the liver. Two types are recognized: erythropoietic and hepatic.
- Precision:** The ability to reproduce a result.
- Primary fibrinolysis:** Activation of the fibrinolytic system that is not secondary to coagulation.
- Primary hemostasis:** The interaction of platelets and the vascular endothelium to stop bleeding following vascular injury.
- Primary myelofibrosis (PMF):** Also called idiopathic myelofibrosis or idiopathic myeloid metaplasia with

myelofibrosis, is a clonal hematopoietic stem cell disorder characterized by bone marrow fibrosis, symptom burden, splenomegaly, and cytopenias.

Proaccelerin: Factor V; it functions in the common pathway of coagulation as a cofactor.

Probe: A nucleic acid probe is a tool for identifying a particular nucleotide sequence in DNA or RNA. A probe is composed of a nucleotide sequence that is complementary to the sequence of interest and is therefore capable of hybridizing to that sequence. Probes may be labeled to recognize or to count probe signals using a detector.

Proconvertin: Factor VII; it functions in the extrinsic system of coagulation.

Proliferating pool: Consists of myeloblasts, promyelocytes, and myelocytes. The cells spend 3 to 6 days in this pool and are capable of DNA synthesis and undergo cell division.

Pronormoblast: The first recognizable "mother cell" (precursor) of the erythrocytic cell line. (Synonym: **Rubriblast**.)

Protein C: A vitamin K-dependent serine protease that functions as a major regulatory protein in the control of coagulation.

Protein S: A vitamin K-dependent factor that is a necessary cofactor in the reaction in which factor Va is inactivated by protein C.

Prothrombin: Also known as factor II (F II), is a single-chain glycoprotein synthesized in the liver that is the most abundant clotting factor, has the longest half-life of the vitamin K-dependent clotting proteins, and circulates as a zymogen to the serine protease thrombin (Factor IIa).

Prothrombinase complex: The association of factor Xa, factor V, phospholipid, and Ca^{2+} , due to enzymatically converting the substrate prothrombin to the enzymatically active thrombin. Also known as the prothrombin activator.

Prothrombin group: Consists of low molecular weight proteins that include factors II (prothrombin), VII (stable factor), IX (Christmas factor), and X (Stuart-Prower factor).

Prothrombin time (PT): A test to evaluate the overall integrity of the clotting system that involves factors VII, X, V, II, and I. Commonly referred to as a means of evaluating the extrinsic system of coagulation.

Protoporphyrin: A porphyrin whose iron complex forms the heme of hemoglobin and the prosthetic groups of myoglobin and certain respiratory pigments.

Pseudohypochromia: Hypochromia in which the area of pallor is distinctly outlined.

Pyknosis (pyknotic): Condensation and shrinkage of cells through degeneration.

Pyruvate kinase (PK): An essential enzyme in RBCs that generate adenosine triphosphate (ATP) through the Embden-Meyerhof glycolytic pathway.

Pyruvate kinase deficiency: An enzymatic disorder in the Embden-Meyerhof pathway caused by a deficiency in pyruvate kinase. Hemolysis and anemia persist after splenectomy. The trait is autosomal recessive.

Q

Quality Assurance: The activities implemented in a quality plan so that quality requirements for a product or service will be fulfilled.

Quality Control: The observation and review techniques used to fulfill requirements for quality.

Quality control levels: The observation and review techniques used to fulfill requirements for quality.

Quality System Essentials (QSEs): A road map for how a laboratory establishes a Quality Management System (QMS).

R

Reflex test: A parameter that automatically requests further testing.

Reiter's syndrome: A reactive arthritis caused by intestinal bacteria that also affect the skin, eyes, and muscles.

Replication: The process by which DNA is copied. Replication is carried out by the enzyme *DNA polymerase* that recognizes single stranded DNA and fills in the appropriate complementary nucleotides to produce double stranded DNA. Synthesis is initiated at a free 5' end where double stranded DNA lies adjacent to single stranded DNA, and replication proceeds in the 5' direction. In the laboratory, DNA replication is induced as a means of copying DNA sequences.

Reptilase: An enzyme, thrombin-like in nature, derived from the venom of *Bothrops atrox*. It predominantly hydrolyzes fibrinopeptide A from the fibrinogen molecule, in contrast to thrombin, which hydrolyzes fibrinopeptides A and B.

Restriction endonuclease: An enzyme that cleaves DNA at specific nucleotide sequences. For example, *HindIII* cleaves DNA only where the sequence 5'-AAGCTT-3' is present. A variety of other enzymes cut at different specific target sequences.

Reticulocyte: A red blood cell containing a network of granules or filaments representing an immature stage in development. It normally comprises about 1% of circulating red blood cells.

Reticuloendothelial system (RES): A term applied to those cells scattered throughout the body that have the power to ingest particulate matter. It includes histiocytes of loose connective tissue; reticular cells of lymphatic organs; Kupffer cells of the liver; cells lining blood sinuses of the spleen, bone marrow, adrenal cortex, and hypophysis; and other cells.

Ribonucleic acid (RNA): A nucleic acid that controls protein synthesis in all living cells. There are three different types, and all are derived from the information encoded in the DNA of the cell. Messenger RNA (mRNA) carries the code for specific amino acid sequences from the DNA to the cytoplasm for protein synthesis. Transfer RNA (tRNA) carries the amino acid groups to the ribosome for protein synthesis. Ribosomal RNA (rRNA) exists within the ribosomes and is thought to assist in protein synthesis.

Ring sideroblasts: Nucleated red blood cells in which iron is accumulated in the mitochondria that surround the nucleus.

Ristocetin cofactor activity assay: A method that quantitatively measures plasma vWF activity and also utilizes an aggregometer to determine the rate of agglutination of a standardized suspension of formalin-fixed normal platelets, ristocetin (1.0 mg/mL), and patient plasma.

Ristocetin-induced platelet agglutination (RIPA) assay: A method that qualitatively assesses the platelet-vWF functional interaction and utilizes an aggregometer and evaluates vWF binding with GP Ib/IX/V.

RNA (ribonucleic acid): A single stranded molecule composed of ribonucleotides (A,C,G and U=uracil). RNA is produced by transcribing from a DNA template (a gene), and RNA in turn serves as a template for protein translation.

Romanowsky-stained blood smear: Visualizes Pappenheimer bodies by staining the protein matrix of the granule.

Rouleaux: A group of red blood corpuscles arranged like a roll of coins, owing to an abnormal protein coating on the cells' surfaces; seen in multiple myeloma and Waldenström's macroglobulinemia.

Run: A finite length of time or number of patient samples. QC is designed to be analyzed at the beginning and end of a run. The CLIA definition of a run for QC purposes is 24 hours for most analytes. However, coagulation and a few other tests require QC analysis every 8 hours.

5

Schistocyte: An abnormal red cell that is formed when pieces of the red cell membrane become fragmented. Whole pieces of the red cell membrane appear to be missing, causing bizarre-looking red cells.

Secondary hemostasis: The enzymatic activation of the coagulation proteins to produce fibrin from fibrinogen.

Segmented neutrophils: Mature granulocytes.

Senescence: The aging process of the red cells.

Serine proteases: A family of proteolytic enzymes with the amino acid serine at the active site.

Serous fluid: Fluid from the thoracic and abdominal cavities.

Sézary syndrome: Skin disease characterized by infiltration with atypical Sézary cells. The exfoliative dermatitis is considered a variant form of mycosis fungoides.

Shift: An abrupt move in which six or more consecutive QC values above or below the mean.

Shift to the left: An abnormal cell maturation situation that occurs when increased bands, less mature neutrophils, and a smaller average number of lobes are found in segmented cells; it may be caused by infection, hematologic disorders, or physiologic factors.

Shift to the right: An abnormal cell maturation situation that occurs when more than one hypersegmented cell is seen; it is indicative of vitamin B12 or folate deficiency.

Sickle cell anemia (HbSS disease): A hereditary, chronic anemia in which abnormal sickle- or crescent-shaped erythrocytes are present. It is caused by the presence of hemoglobin S in the red blood cells. The gene that causes this disease occurs with high frequency in African and Mediterranean populations.

Sideroblast: A ferritin-containing normoblast in the bone marrow. It makes up from 20% to 90% of normoblasts in the marrow.

Siderophages: Iron-containing macrophages.

Siderotic granules: Iron-containing granules. See Pappenheimer bodies.

Sinuses: An artery entering the bone branches out toward the periphery to specialized vascular spaces.

Six Sigma approach: A quality plan based on reducing error to achieve quality results.

Small lymphocytic lymphoma (SLL): The tissue equivalent of chronic lymphocytic leukemia (CLL) in the marrow and blood with a diffuse growth pattern, although pseudofollicular growth centers may be observed.

Spectrin: A large molecule, found on the inner surface of red blood cell membrane, that is responsible for the biconcave shape of the red cell as well as for its deformability.

S-phase fraction (SPF): Percentage of cells that are replicating their DNA. The higher the S-phase fraction the more rapidly cells are dividing and the more aggressive is a malignancy.

Spherocyte: An abnormal red blood cell shape that appears as a solid reddish-orange disc with no central pallor. Spherocytes are smaller than normal red cells, have a concentrated hemoglobin content, and have a decreased surface-to-volume ratio.

Spillover: The overlap of the emission spectra of two fluorophores.

Splenomegaly: Enlargement of the spleen seen in several blood disorders.

Staining Index (SI): Used to estimate the brightness of a fluorophore through the relationship between the positive and negative signals.

Standard deviation (SD): Mathematically describes the spread of data about the mean.

Statistical process control: A general term for the parts of a control system in which statistics are used, such as a Levy-Jennings graph

Stoke's Shift: The difference, in nanometers, between the peak excitation and the peak emission wavelengths. Each fluorophore has a distinct and individual Stoke's Shift.

Stomatocyte: An abnormal red cell shape; this shape appears as having a slitlike area of central pallor.

Streptokinase: A product of beta-hemolytic streptococci capable of liquefying fibrin.

Subarachnoid space: The area between the arachnoid mater and pia mater.

Syncytium: A mass of cytoplasm containing several nuclei.

Synovial cavity: The space that exists between the bones and is enclosed by the synovium and intra-articular cartilage.

Synovial fluid (mucin): An ultrafiltrate of plasma secreted by the synovial cells and transports nutrients to the articular cartilage.

T

Tandem Dyes: A fluorescent dye that consists of two coupled fluorophores. Light excitation of one of these fluorophores causes it to emit light that excites the other.

Target cells: A cell that resembles a target (sometimes referred to as a "bull's eye" cell) that appear on the

peripheral blood because of an increase in RBC surface membrane. (Synonym: **Codocyte**.)

Teardrop cells: An abnormal red cell, shaped like a tear, seen frequently in the myeloproliferative disorders. (Synonym: **Dacrocyte**.)

Thalassemia: A group of hereditary anemias produced by either a defective production rate of alpha- or beta-hemoglobin polypeptide. This disorder is inherited in homozygous or heterozygous state.

Thalassemia major: The homozygous form of deficient beta chain synthesis, which is very severe and presents itself during childhood. Prognosis varies; however, the younger the child when the disease appears, the more unfavorable the outcome.

Thrombin: An enzyme that converts fibrinogen to fibrin so that a soluble clot can be formed.

Thrombin time (TT): A coagulation procedure that measures the time required for thrombin to convert fibrinogen to an insoluble fibrin clot.

Thrombocytopenia: Decreased numbers of platelets.

Thrombocytosis: Increased numbers of platelets.

Thrombotic thrombocytopenic purpura (TTP): A severe condition characterized by thrombocytopenia, microangiopathic hemolytic anemia, renal dysfunction, neurologic abnormalities, and fever.

Tissue factor (TF): An endothelial cell phospholipid that forms a complex with factor VIIa to activate factor X.

T. factor pathway inhibitor: A plasma protein that inhibits its tissue factor-factor VIIa complex in the extrinsic clotting cascade.

Tissue plasminogen activator (tPA): A clotting factor produced by vascular endothelial cells that selectively bind to fibrin as it activates fibrin-bound plasminogen.

Toxic granulation: Medium to large metachromatic granules that are evenly distributed throughout the cytoplasm. May be seen in severe bacterial infections, severe burns, and other conditions.

Toxic vacuolization: Round, clear unstained areas that are dispersed randomly throughout the cytoplasm of neutrophils in patients with overwhelming infections. Strongly indicates a serious bacterial infection.

Trabeculae: Bands or bundles of connective tissue.

Transcription: Synthesis of RNA from a DNA template (a gene).

Transferrin: A glycoprotein synthesized in the liver, with the primary function of iron transport.

Transferrin Receptor: Membrane glycoprotein that mediates cellular uptake of ferric transferrin.

Translation: Synthesis of protein from an RNA template (a transcript).

Translocation: A chromosomal rearrangement whereby part of one chromosome breaks off and becomes attached to another chromosome. The site of juxtaposition between the two chromosomes is referred to as the breakpoint.

Trend: A slow change in QC values on a QC chart, either rising or falling steadily by a set of six or more consecutive data points.

U

Unassayed Control Material: A commercially prepared control that does not have specific ranges verified by the manufacturer. The laboratory must run the control material in replicate over several days or weeks to establish an initial mean and SD.

Urokinase: A trypsin-like protease, found in the urine and synthesized by the kidney, that activates plasminogen by proteolytic cleavage. Differs from tissue plasminogen activators in that urokinase reacts with plasminogen in the fluid phase of blood.

V

VCS technology: A combination of cell volume, conductivity, and light scatter measurements used to differentiate white blood cells and enumerate nucleated red blood cells (NRBCs).

Venous thromboembolism (VTE): A condition that occurs when a blood clot forms in a vein.

Ventriculoperitoneal shunt: A drainage device inserted into the ventricles of the brain to remove excess CSF in cases of hydrocephalus, neoplastic conditions, or head injury.

Viscous metamorphosis: The transformation of irreversibly aggregated platelets into a mass of degenerative platelet material without membranes.

vWF-binding activity assay (vWF: Co): Assesses the ability of vWF to bind to collagen.

von Willebrand disease (vWD): A congenital bleeding disorder inherited as an autosomal-dominant trait and characterized by a decreased level of factor VIII:C and a prolonged bleeding time

von Willebrand factor (vWF): A component of the factor VIII molecule that mediates platelet interaction with subendothelium.

W

Westgard Rules: A set of rules to assist the technologist in making decisions when reviewing QC data.

White blood cell (WBC): Also known as leukocytes, protect the body from infection.

World Health Organization (WHO): A specialized agency of the United Nations responsible for international public health.

X

Xanthochromia: A pink, orange, or yellow color of the supernatant, caused by the breakdown of a hemoglobin.

Xerocytosis: A decrease in red cell hydration and volume due to abnormalities in red cell cation permeability.

Z

Z-score: Measures how many standard deviations the mean of an analyte is from the mean of the peer group. A Z-score has no units; however, the score can be positive or negative.

Zymogen: A substance that, when paired with its zymase, becomes an enzyme.

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Hematologic Values*

Determination	Reference Range		Notes
	Conventional	SI	
Hematology			
"Complete" blood count (CBC): Automated Methodology			
WBC	4.5–11.0 × 10 ³ /μL	4.5–11.0 × 10 ⁹ /L	
RBC	Male: 4.7–6.1 × 10 ⁶ /μL Female: 4.2–5.4 × 10 ⁶ /μL	4.7–6.1 × 10 ¹² /L 4.2–5.4 × 10 ¹² /L	
Hemoglobin (Hgb)	Male: 13.5–17.5 g/dL Female: 12–16 g/dL Infant: 14–22 g/dL	135–175 g/L 120–160 g/L 140–220 g/L	Direct measurement
Hematocrit (Hct)	Male: 42%–52% Female: 37%–47%	0.42–0.52 L/L 0.37–0.47 L/L	Calculation: RBC × MCV/10
Mean corpuscular volume (MCV)	80–100 fL	80–100 fL	Derived from RBC histogram: Measured volume of 1 RBC Calculation: Hct/RBC × 10
Mean corpuscular hemoglobin (MCH)	27–34 pg	27–34 pg/cell	Calculation: Hgb/RBC × 10
Mean corpuscular hemoglobin concentration (MCHC)	32%–36%	320–360 g/L 320–360 g/L	Calculation: Hgb/Hct × 100
Red cell distribution width (RDW)	11.5%–14.5%		Derived from RBC histogram:
Platelets (PLT)	150,000–450,000/μL	150–450 × 10 ⁹ /L	
Mean platelet volume (MPV)	7.4–10.4 fL	7.4–10.4 fL	Derived from PLT histogram
WBC Differential			
Lymphocytes	20%–44%		
Monocytes	2%–9%		
Neutrophils	50%–70%		
Bands	2%–6%		
Eosinophils	0%–4%		
Basophils	0%–2%		
Absolute Counts			
Lymphocytes	1.2–4.8 × 10 ³ /μL	1.2–4.8 × 10 ⁹ /L	Lymphs %/100 × WBC
Monocytes	0.2–0.8 × 10 ³ /μL	0.2–0.8 × 10 ⁹ /L	Mono %/100 × WBC
Neutrophils	1.5–7.7 × 10 ³ /μL	1.5–7.7 × 10 ⁹ /L	Neutrophils %/100 × WBC
Bands	0–0.7 × 10 ³ /μL	0–0.7 × 10 ⁹ /L	Bands %/100 × WBC
Eosinophils	0–0.5 × 10 ³ /μL	0–0.5 × 10 ⁹ /L	Eos %/100 × WBC
Basophils	0–0.2 × 10 ³ /μL	0–0.2 × 10 ⁹ /L	Baso %/100 × WBC

*Please note: Normal values vary by institution, patient population, and testing methodology.

Continued

Hematologic Values*—cont'd

Determination	Reference Range		Notes
	Conventional	SI	
Reticulocyte count	Newborn: 2.5%–6.0% Adult: 0.5%–2.5%		Calculation: %Retic × RBC
Absolute reticulocyte	24,000–84,000/μL	24–84 × 10 ⁹ /L	
Reticulocyte hemoglobin (Ret-He)	27–33 pg/cell		
Erythrocyte sedimentation rate (ESR)	Male: 0–22 mm/hr Female: 0–29 mm/hr		Westergren method
RBC Enzymes			
G6PD	8.0–16.5 U/g Hgb	516–1064U/mmol Hgb	
Pyruvate kinase	5.0–11.5 U/g Hgb	322–742U/mmol Hgb	
Iron Studies			
Serum iron	Adult male: 65–170 μg/dL Adult female: 50–170 μg/dL	11.63–30.43 μmol/L 8.95–30.43 μmol/L	
TIBC	250–450 μg/dL	44.75–80.55 μmol/L	
Transferrin saturation	Male: 20%–50% Female: 15%–50%		
Serum sTfR (soluble transferrin receptor)	1.5–2.75 mg/L	17.6–32.4 nmol/L	
Serum transferrin	Adult 200–360 mg/dL Adult >60 yrs: 160–340 mg/dL	2.0–3.6 g/L 1.6–3.4 g/L	
Ferritin (serum)	Adult male 20–250 ng/mL Adult female 10–120 ng/mL	20–250 μg/L 10–120 μg/L	
Zinc protoporphyrin (free erythrocyte protoporphyrin)	16–65 μg/dL	0.28 μmol/L–1.17 μmol/L	
Folic Acid			
Normal	2.6–12.2 μg/L	5.9–27.6 nmol/L	
Red cell folate	150–450 μg/L	340–1020 nmol/L	
Haptoglobin	30–200 mg/dL	0.3–2.0 g/L	

SI = System of International Units; G6PD = glucose-6-phosphate dehydrogenase deficiency; TIBC = total iron-binding capacity
 *Please note: Normal values vary by institution, patient population, and testing methodology.

Hematologic Values

Determination	Reference Range		Notes
	Conventional	SI	
Hemoglobin (Hgb) Studies			
Electrophoresis			
A ₁ hemoglobin	95%–97%		
A ₂ hemoglobin	2%–3%		
Fetal hemoglobin	1%–2%		
Hemoglobin, met- Cytochrome b5 reductase (CYB5R) or methemoglobin reductase	<2.0% of total Hgb 7.8–13.1 U/g Hgb		
Hemoglobin, sulf-	0.0%–1.0% of total Hgb		
Carboxy hemoglobin	0.0%–1.4% of total Hgb		
Plasma hemoglobin	0.0–9.7 mg/dL	0.0–0.097 g/L	
Other Red Cell Studies			
Vitamin B ₁₂			
Normal (serum)	180–914 pg/mL	150–616 pmol/L	
Borderline	140–204 pg/mL	102.6–149 pmol/L	
Serum Erythropoietin	4–27 mU/mL	4–27 IU/L	
Leukocyte alkaline phosphatase (LAP)	Male 22–124 Female 33–149		
Score			
Serum Acid phosphatase	0.0–4.3 U/L	0.0–.073 μ kat/L	
Coagulation			
Platelet function tests			
Platelet aggregation	Full response to ADP, collagen, epinephrine, thrombin, and ristocetin		
PFA 100 (closure time)	Collagen/epinephrine <175 sec.		
Coagulation Factors			
One-stage quantitative assay for factors II, V, VII, and X	50%–150% of normal mean		
One-stage quantitative assay for factors VIII, IX, XI, XII	50%–150% of normal mean 50–150 IU/dL		
Factor I (fibrinogen)	180–400 mg/dL	1.8–4.0 g/L	
Factor XIII	70–130% of normal mean		
Factor VIII assay (functional)	50%–200% of normal mean		
Factor VIII inhibitor	0.0–0.5 Bethesda units		

Continued

Hematologic Values—cont'd

Determination	Reference Range		Notes
	Conventional	SI	
Von Willebrand factor (VWF) activity	50-200% of normal mean		
VWF antigen	50-200 IU/dL		
Prothrombin time (PT)	11-13.5 sec (INR of 0.8-1.1)		
Activated partial thromboplastin time (APTT)	21-35 sec		
<i>Fibrinolytic Studies</i>			
D-Dimer test	0.0-0.4 µg/mL	0.0-400 ng/mL	
Thrombin time	12-19 sec		
Reptilase time	15-19 sec		
plasminogen	80-120% of normal mean 150-250 ng/L		
α2-antiplasmin	80-120% of normal mean 0.8-1.2 IU/mL		
Antithrombin	80-120% of normal mean		
Protein C	70-150% of normal mean Adult: 64-128 IU/dL		
Activated Protein C Resistance (APCR)	APC ratio >2.1		
Protein S	70-150% of normal mean Adult: 60-113 IU/dL		
<i>Body Fluids</i>			
Spinal fluid (CSF)	Colorless		
Adults	0-5 mononuclear cells/µL 0 RBCs/µL		Hemocytometer
Neonates	0-30 mononuclear cells/µL 0 RBCs/µL		
<i>Differential</i>			
Adults	Lymphs 28%-96%		Wright stain
	Mono 16%-56%		Cytospin
	Segs 0%-5%		
	Histiocytes rare		
	Ependymal cells rare		
	Eosinophils rare		

Hematologic Values—cont'd

Determination	Reference Range		Notes
	Conventional	SI	
Neonates	Lymphs 2%–38%		
	Mono 50%–94%		
	Segs 0%–5%		
	Histiocytes 1%–6%		
	Ependymal cells rare		
	Eosinophils rare		
Synovial fluid	Pale yellow to colorless		
	Crystals (none)		
	No clot formation		
	200–600 WBCs/ μ L		Hemacytometer
	0 RBCs/ μ L		
	Few synovial cells		
Differential	PMN 0%–25%		Wright stain
	Mononuclear cells 0%–75%		Cytospin method
Pleural/peritoneal fluid	Yellow		
	≤ 1000 WBCs/ μ L		Hemacytometer
	0 RBCs/ μ L		
Differential	PMN δ 25%		
	Mononuclear cells 0%–75%		
Seminal fluid	Total volume: 1.5–5.0 mL		
	Total count: 20–160 mil/mL		Hemacytometer

SI = System of International Units; INR = international normalized ratio;